YEAST PHOSPHOGLYCERATE MUTASE STUDIED BY SITE-DIRECTED MUTAGENESIS.

Malcolm F. White. BSc.Hons.

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2. Materials and methods

DECLARATION

I hereby declare that this thesis has been composed by me, that it has not been accepted in any previous application for a degree and that the work of which it is a record has been carried out by me.

MALCOLM F. WHITE

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ABBREVIATIONS

(1) Miscellaneous

A	-	Adenine
ADP	-	Adenosine-5'-diphosphate
AMP	-	Adenosine-5'-monophosphate
ATP	-	Adenosine-5'-triphosphate
bp	-	base pairs
BPGA	-	Bisphosphoglyceric acid
C	-	Cytosine
C-	-	Carboxy-
CTP	-	Cytosine-5'-triphosphate
d-	-	deoxy-
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
ds	-	double stranded
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
G	-	Guanine
GTP	-	Guanine-5'-triphosphate
HPLC	-	High pressure liquid chromatography
kb	-	kilobases
kbp	-	kilobase pairs
Km	-	Michaelis constant
M_r	-	Relative molecular mass
N-	-	Amino-
NADH	-	Nicotinamide-adenine dinucleotide reduced
NMR	-	Nuclear magnetic resonance
NTP	-	Nucleotide triphosphates
OD	-	Optical density
ORF	-	Open reading frame
PAGE	-	Polyacrylamide gel electrophoresis
PEG	-	Polyethyleneglycol
PGA	-	Phosphoglyceric acid
R_F	-	Relative band speed
rpm	-	revolutions per minute
RT	-	Room temperature

SDS	-	Sodium dodecyl sulphate
SDW	-	Sterile distilled water
ss	-	single stranded
T	-	Thymine
Tris	-	Tris(hydroxymethyl)aminomethane
TTP	-	Thymine-5'-triphosphate
UAS	-	Upstream activating sequence
UV	-	Ultraviolet
w-t	-	wild-type
yeast	-	Saccharomyces cerevisiae

(2) Amino acids

A	-	Ala	-	Alanine
C	-	Cys	-	Cysteine
D	-	Asp	-	Aspartic acid
E	-	Glu	-	Glutamic acid
F	-	Phe	-	Phenylalanine
G	-	Gly	-	Glycine
H	-	His	-	Histidine
I	-	Ile	-	Isoleucine
K	-	Lys	-	Lysine
L	-	Leu	-	Leucine
M	-	Met	-	Methionine
N	-	Asn	-	Asparagine
P	-	Pro	-	Proline
Q	-	Gln	-	Glutamine
R	-	Arg	-	Arginine
S	-	Ser	-	Serine
T	-	Thr	-	Threonine
V	-	Val	-	Valine
W	-	Trp	-	Tryptophan
Y	-	Tyr	-	Tyrosine
		Asx	-	Aspartic acid or asparagine
		Glx	-	Glutamic acid or glutamine

(3) Proteins

BSA	-	Bovine serum albumin
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
LDH	-	Lactate dehydrogenase
PGK	-	Phosphoglycerate kinase
PK	-	Pyruvate kinase

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ABSTRACT

The enzyme phosphoglycerate mutase (EC 5.4.2.1) catalyses the interconversion of 2-phosphoglycerate and 3-phosphoglycerate in the glycolytic/gluconeogenic pathways. It also possesses minor 2,3-bisphosphoglycerate synthase and phosphatase activities. The enzyme from Saccharomyces cerevisiae, whose amino acid sequence and high resolution crystal structure are known, has been particularly well characterised. A detailed catalytic mechanism has been proposed, based on structural and kinetic evidence. The enzyme has a flexible C-terminal 'tail', which is essential for its activity. The closely related enzyme bisphosphoglycerate synthase (EC 5.4.2.4/3.1.3.13), which is present in vertebrate erythrocytes, catalyses the same three reactions, but has a much lower mutase activity and a higher synthase activity. The two enzymes share about 50% sequence identity. The aim of this project was to probe the molecular basis for the mutase/synthase relationship and the function of the C-terminal tail using protein engineering of the S.cerevisiae enzyme.

The gene encoding S.cerevisiae phosphoglycerate mutase (GPM) was located and sequenced. A strain of yeast, entitled DBYgpm-, was constructed in which the chromosomal copy of the GPM gene has been deleted. Two mutant forms of the enzyme were designed and constructed, expressed in DBYgpm-, purified and kinetically characterised. The first, in which the C-terminal lysine had been replaced by a glycine (K246G), was not significantly different kinetically from the wild-type enzyme. This suggests that the C-terminal lysine plays no major part in catalysis. In the second, an active site serine had been replaced by a glycine (S11G). In the S.cerevisiae enzyme this residue is thought to be important as a phospho-ligand, whereas in the synthase enzyme it is a glycine and could play no part in ligand binding. For the S11G mutant, the affinity for 2,3-bisphosphoglycerate was lowered tenfold and the mutase activity was decreased by 99.5% whilst the synthase activity was unaffected. These observations confirm the important role of serine 11 in the binding of 2,3-bisphosphoglycerate and in the mutase but not the synthase reaction.

1. Introduction.

1.1 Glycolysis

The pathway of glycolysis (figure 1.1) is an ancient one, and is found, at least in part, in all living organisms. The glycolytic enzymes exist at relatively high levels in most cells. They have been extensively characterized both structurally and kinetically. The amino acid sequences of the enzymes have been determined from a number of different sources, and are found to be amongst the most highly conserved proteins (Fothergill-Gilmore, 1986). The crystal structures of most have been determined, and share a common structural motif - a core of ß-strands surrounded by α-helices.

The advent of recombinant DNA technology has added a fresh impetus to the study of this pathway. The development of a simple method for DNA sequencing has led to many more protein sequences being determined. More recently, the development of site-directed mutagenesis has allowed ideas on the molecular basis for activity and specificity of several glycolytic enzymes to be tested directly.

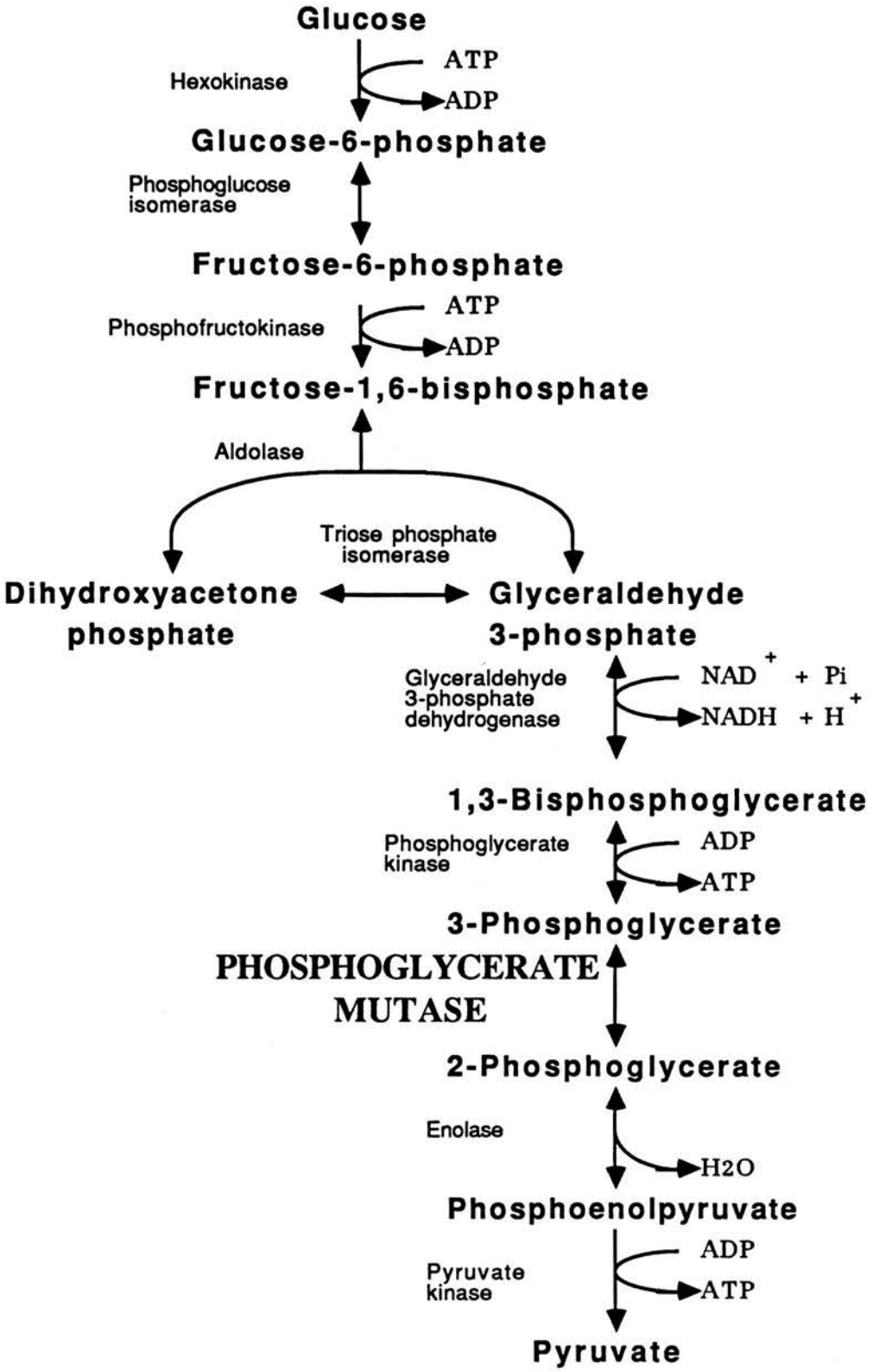

Figure 1.1. The pathway of glycolysis. The reaction catalysed by phosphoglycerate mutase is indicated.

1.2 The phosphoglycerate mutases

Phosphoglycerate mutase catalyses the interconversion of 2- and 3-phosphoglycerate in the lower half of the glycolytic pathway. The enzyme was first identified over 50 years ago (Meyerhof and Kiessling, 1935), and has since been detected in every organism so far studied, with the possible exception of certain thermophilic archaebacteria (Budgen and Danson, 1986). Two main types of mutase are known - one which requires the cofactor 2,3-bisphosphoglycerate (2,3-BPGA) for activity and is thus termed 'cofactor-dependent'; and the other, which has no requirement for 2,3-BPGA and is termed 'cofactor-independent'.

1.3 Distribution of cofactor-dependent and cofactor-independent mutases

Cofactor-independent mutases were at first thought to be confined to higher plants, however the subsequent extensive screening of over 80 species revealed a much more complex distribution. Thus plants, filamentous fungi, invertebrates such as sponges, sea-urchins and spiders and the Gram positive Bacillus bacteria have been shown to possess cofactor-independent mutases (Price et al., 1983; Carreras et al., 1982; Singh and Setlow, 1979; Watabe and Freese, 1979) whilst cofactor-dependent enzymes are found in vertebrates, certain invertebrates such as mussels, earthworms and insects (Carreras et al., 1982), fungi such as Saccharomyces cerevisiae and Schizosaccharomyces pombe (Price et al., 1983) and Gram negative bacteria such as Escherichia coli (D'Alessio and

Josse, 1971) and Zymomonas mobilis (Pawluk et al., 1986). Cofactor-dependent phosphoglycerate mutase can be distinguished from the cofactor-independent form by its ability to bind to cibacron blue-Sepharose and to be eluted by the cofactor 2,3-BPGA (Price and Stevens, 1983). If one accepts this property as diagnostic of cofactor-dependent enzymes, then studies of Gram positive lactic acid bacteria by (Kawai et al., 1981) uncover a further layer of complexity in the distribution of the two enzymes. Using the dye-binding/elution test, they found that four Lactobacillus species, two Streptococcus species and two Pediococcus species possessed cofactor-independent enzymes, whilst four Lactobacillus species had cofactor-dependent enzymes. Furthermore, in the species Leuconostoc mesenteroides, two strains tested were cofactor-dependent and two cofactor-independent.

The complexity of the distribution of the cofactor-dependent and cofactor-independent enzymes suggests that the two genes which code for them were both present early in evolution and have been inherited in a haphazard fashion.

1.4 Cofactor-independent phosphoglycerate mutase

The cofactor-independent mutases are inherently unstable enzymes and have proved recalcitrant to purification. Consequently comparatively little is known about this class of the enzyme. A cofactor-independent mutase was first identified in wheat-germ (Ito and Grisolia, 1959) and has since been noted in a number of organisms. The enzyme appears to be a monomer, M_r about 60,000,

with a pH optimum around 8.5 (Fothergill-Gilmore and Watson, 1989). Isotope exchange experiments have shown that it is capable of transferring a phospho-group directly to glycerate at the active site (Breathnach and Knowles, 1977). Recently two distinct isoenzymes have been identified in certain higher plants, corresponding to cytosolic and plastid forms of the enzyme. The cytosolic form is more abundant and has a higher affinity for substrate (Botha and Dennis, 1986). The enzyme from Bacillus species is a cofactor-independent monomer, with a similar M_r, but differs from all other mutases in exhibiting an absolute requirement for Mn^{2+} (Singh and Setlow, 1979; Watabe and Freese, 1979). A lack of any protein sequence information limits the further analysis of the cofactor-independent enzyme.

1.5 Cofactor-dependent phosphoglycerate mutases

The cofactor-dependent mutases have proved much easier to purify, and thus have been much more extensively characterised. The enzyme exhibits a varied quaternary structure: tetrameric in yeast such as S.cerevisiae; dimeric in vertebrates and Gram negative bacteria such as E.coli (D'Alessio and Josse, 1971) and Z.mobilis (Pawluk et al., 1986) and in some Gram positive bacteria such as Leuconostoc dextranicum (Kawai et al., 1983); and monomeric in the fission yeast S.pombe (Price et al., 1985a). The subunit M_r is commonly in the range 27,000-30,000, although the S.pombe enzyme appears to be smaller at 23,000 (for a review, see Fothergill-Gilmore and Watson, 1989).

A great deal of effort has gone into the study of all aspects of cofactor-dependent phosphoglycerate mutases, particularly those from vertebrates and S.cerevisiae. The remainder of this introduction will concentrate on aspects of the structure, activities, molecular mechanism and evolution of the cofactor-dependent mutases.

1.6 Evolution of phosphoglycerate mutases

It is apparent that two quite distinct enzymes with phosphoglycerate mutase activity exist. These two enzymes are probably not homologous and may both have existed in an ancestral organism; one or the other being lost in the course of evolution to give the complex distribution we see today (Fothergill-Gilmore and Watson, 1989).

1.6.1 The M and B isoenzymes

The gene inherited by early vertebrates, coding for a cofactor-dependent enzyme, appears to have undergone a series of gene duplication events. Fish and all higher vertebrates (excluding birds) possess two isoenzymes of differing mobility in cellulose acetate electrophoresis (Mezquita and Carreras, 1981). These have been termed the B (brain) and M (muscle) isoenzymes, the B form being the more mobile of the two. In mammals, the B form is present in most tissues excluding skeletal muscle, whilst the M form is only found in cardiac and skeletal muscle. In tissues where both isoenzymes are expressed, for example in the heart, MB heterodimers are observed (Bartrons and Carreras, 1982). The electrophoretic mobility, heat stability and sensitivity to -SH group modification of the isoenzymes from a variety of species have been investigated, and are summarised in table 1.1.

Table 1.1. Physical properties of vertebrate phosphoglycerate mutases

All data and experimental details can be found in (Mezquita et al., 1981). Isoenzymes are designated 'M' or 'B' according to their observed electrophoretic mobility. 'nd' indicates a property was not determined.

organism	isoenzyme	sensitive to heat	sensitive to -SH modn.	electrophor. mobility
mammals	M	-	+	slow
	B	+	-	fast
reptiles	M	-	+	slow
	B	+	-	fast
pigeon	B	+	-	fast
chicken	B	+	+	fast
amphibians	M	nd	+	slow
	B	nd	+	fast
fish	M	nd	+	slow
	B	nd	+	fast

The two isoenzymes share around 80% sequence identity in humans (Sakoda et al., 1988) and display very similar kinetic properties (Berrocal and Carreras, 1982). The differing sensitivity to -SH group modifying agents can be explained by the active site residue 20, which is a cysteine in the M isoenzyme and a threonine in the B isoenzyme. Interestingly, the enzymes present in pigeon and chicken differ in their sensitivity to -SH modification, presumably for the same reason. In the lower vertebrates, amphibians and fish, both the M and B isoenzymes are sensitive, although this is

not the case for the enzyme from S.cerevisiae, which has no cysteine residues. Can we say which is the ancestral form of the enzyme? The B isoenzyme appears to be more widely expressed in vertebrate tissues, indeed the electrophoretic evidence suggests that birds appear to possess only the B isoenzyme (Mezquita and Carreras, 1981). Also, analysis of human genomic DNA has shown that several copies of the B gene exist, possibly in the form of processed pseudogenes (Sakoda et al., 1988). This is perhaps evidence that the M gene may have arisen from a duplication of the B gene, the B form of the enzyme being ancestral.

1.6.2 The E isoenzyme

Another gene duplication event gave rise to a third isoenzyme - E (erythrocyte). The E isoenzyme is expressed mainly in the erythrocytes of most vertebrates, probably from amphibia onwards, where it is responsible for the synthesis of 2,3-BPGA; a potent effector of haemoglobin. This role for 2,3-BPGA probably first arose in amphibia, thus the evolution of this isoenzyme is closely linked with the evolution of haemoglobin as an oxygen transporter (Fothergill-Gilmore and Watson, 1989). The E isoenzyme is also expressed at low levels in other tissues, where it exists in the form of BE and ME heterodimers (Tauler et al., 1986; Tauler and Carreras, 1987). This isoenzyme probably also arose from duplication of the B gene, as the human B and E genes share some limited sequence identity in their downstream non-coding regions (Sakoda et al., 1988). The sequences of three E isoenzymes are

known - from human, rabbit and mouse. They are very highly conserved (90%), and have about 50% identity with the yeast, and human M and B enzymes.

1.6.3 Phylogeny

What was the order of appearance of the three isoenzymes M, B and E? As we have seen, there is reasonable evidence that the B form corresponds to the ancestral one, the M and E isoenzymes arising from duplications of the B gene followed by divergent evolution. There are two schools of thought over the order of appearance of the M and E isoenzymes. One, based on primary structure information, suggests that the B and M isoenzymes, being 80% identical in amino acid sequence, must have diverged later than the E isoenzyme, which is only about 50% identical to either the B or M isoenzyme (Fothergill-Gilmore and Watson, 1989). The other, based primarily on the isoenzyme studies and on the supposed link between the E isoenzyme and haemoglobin discussed above, suggests that the B/M divergence predates the B/E divergence. Whilst there is not enough evidence to reach a firm conclusion, the latter explanation appears more likely at present. The sequence information does not necessarily conflict with this theory as one can consider the E isoenzyme to be functionally distinct from the M and B forms, which appear to have the same role in different tissues and whose divergence may therefore have been more constrained in the course of evolution.

1.7 Reactions catalysed by phosphoglycerate mutases

Phosphoglycerate mutases catalyse three reactions : the interconversion of 2- and 3-phosphoglycerate and the synthesis and degradation of 2,3-bisphosphoglycerate (fig. 1.2). These reactions are commonly called the mutase, synthase and phosphatase activities respectively. Originally it was envisaged that three different enzymes catalysed these three reactions, however multiple activities consistently co-purified from a variety of sources (Rose and Whalen, 1973; Rosa et al., 1975). It was then suggested that all three reactions could be catalysed by a single polypeptide in human erythrocytes and yeast (Sasaki et al., 1975,76). The ancestral enzyme, which corresponds to the yeast and vertebrate M and B enzymes found today, has a very high ratio of mutase : synthase activities, and is commonly denoted 'phosphoglycerate mutase' or 'monophosphoglycerate mutase'. The E isoenzyme, which probably evolved more recently, has a ratio of mutase : synthase activities which approaches parity, and is usually termed 'bisphosphoglycerate mutase' or 'bisphosphoglycerate synthase'. The relationship between these two forms of the enzyme is discussed in more detail in section 1.10.

1.7.1 Investigation of the reaction mechanism

Early experiments demonstrated that the mutase activity of rabbit M-type phosphoglycerate mutase was dependent on catalytic amounts of 2,3-bisphosphoglycerate (Sutherland et al., 1949a), and it was

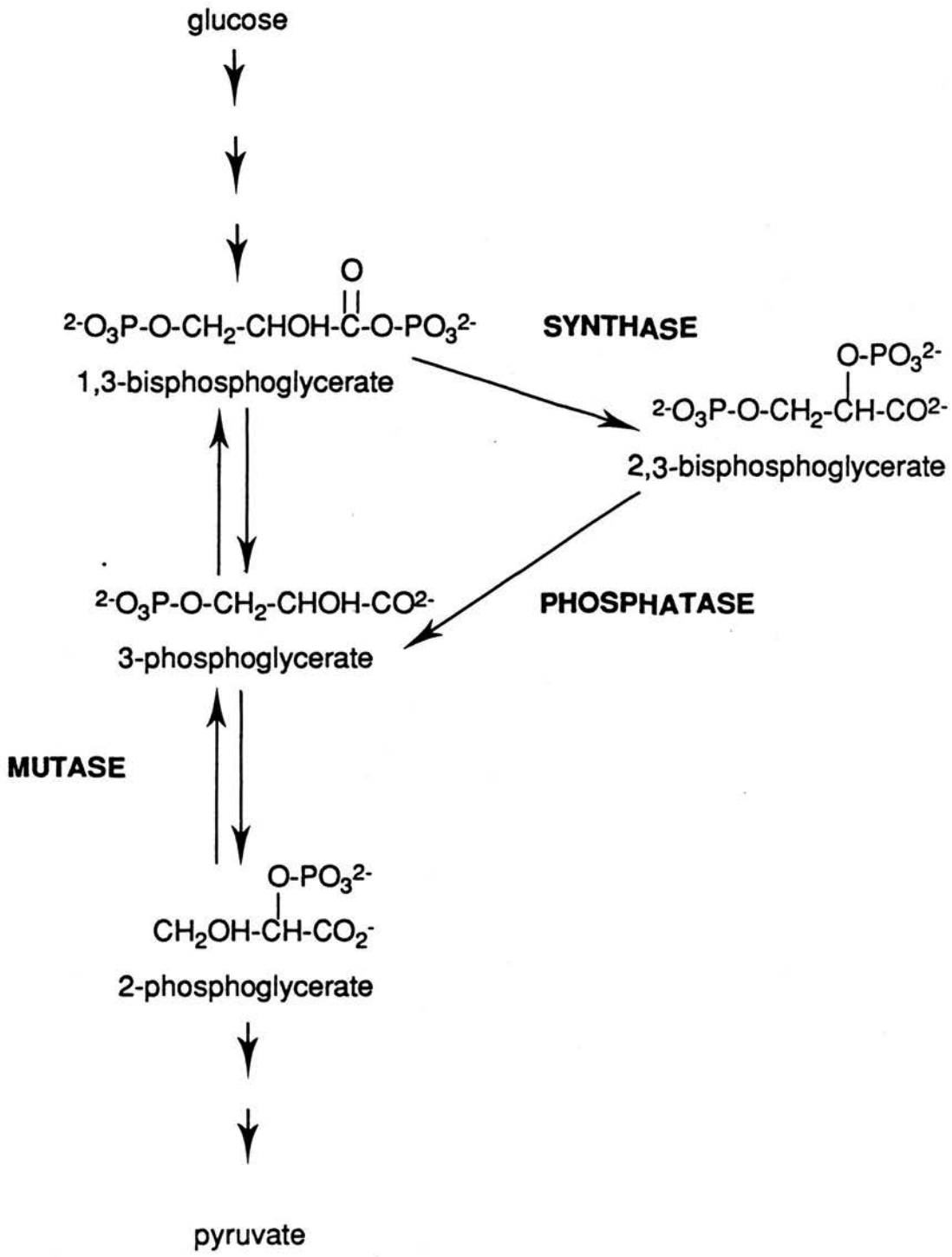

Figure 1.2. The three reactions catalysed by cofactor-dependent phosphoglycerate mutases.

subsequently shown that this cofactor was required only once in every 100 catalytic cycles (Grisolia and Cleland, 1968). In one of the first experiments to use a radioactive tracer, it was shown that after a 30 min incubation of rabbit M-type phosphoglycerate mutase with ^{32}P-labelled 2-phosphoglycerate, the radioactive label could be detected in 3-phosphoglycerate, 2-phosphoglycerate and 2,3-bisphosphoglycerate (Sutherland et al., 1949b). This important experiment demonstrated that all three ligands bound at the active site of the enzyme, and that phospho-transfer could occur between them.

Two possibilities existed for the nature of the phospho-transfer event catalysed by phosphoglycerate mutase. One school of thought predicted the existence of a ternary complex at the active site, with the direct transfer of a phospho-group from 2,3-bisphosphoglycerate to a monophosphoglycerate (Chiba et al., 1970). The other postulated a ping-pong mechanism involving a phospho-enzyme intermediate (Grisolia and Cleland, 1968). Attempts to clarify this situation by kinetic analysis led to conflicting results, but the development of an induced transport test based on flux kinetics proved conclusively that yeast, rabbit M-type and pig B-type phosphoglycerate mutase all functioned by a ping-pong, phosphoenzyme mechanism (Britton et al., 1972a,b, 1973). This mechanism was later found to be consistent with the rates of phosphorylation of the chicken B-type and horse E-type enzymes by 2,3-bisphosphoglycerate. Using a stopped-flow technique, Rose and

Dube (1976) demonstrated that these enzymes were phosphorylated by the cofactor at rates consistent with the overall rates of the mutase reactions which they catalysed (see section 1.10.2).

Assuming that a phospho-enzyme was formed during catalysis, which residue was phosphorylated? Rose (1970,71) identified a histidine residue as becoming phosphorylated after incubation of the yeast or rabbit M-type enzymes with ^{32}P-labelled 2,3-bisphosphoglycerate. Subsequently, the isolation of phospho-peptides from the yeast and human and horse E-type enzymes enabled the identification of the His-8 residue (yeast numbering) as the phosphohistidine (Han and Rose, 1979; Hass et al., 1980).

1.8 Structure of phosphoglycerate mutase

1.8.1 Primary structure

The first primary structure information for phosphoglycerate mutases came from the determination of the amino acid sequences of the yeast and human E-type enzymes by protein sequencing (Fothergill and Harkins, 1982; Haggarty et al., 1983). The two enzymes were shown to share 47% identity, emphasising their close evolutionary relationship. The advent of recombinant DNA technology, and specifically the development of methods for the isolation and rapid DNA sequencing of chromosomal and cDNA copies of specific genes, has recently led to the correction of the yeast and human E enzyme sequences (White and Fothergill-Gilmore, 1988; Joulin et al., 1986), and to the determination of the human M and B and rabbit and mouse E isoenzyme sequences.(Shanske et al., 1987; Blouquit et al., 1988; Yanaga et al., 1986; LeBoulch et al., 1988). The sequences are compared in figure 1.3, and show obvious homology. As we shall see, the availablity of sequence information for a range of phosphoglycerate mutases has been a great aid in elucidating the molecular basis for the activities of the enzymes.

1.8.2 Tertiary structure

The high resolution crystal structure of yeast phosphoglycerate mutase has been determined (Winn et al., 1981). The protein exists as a tetramer, composed of four identical subunits, with almost

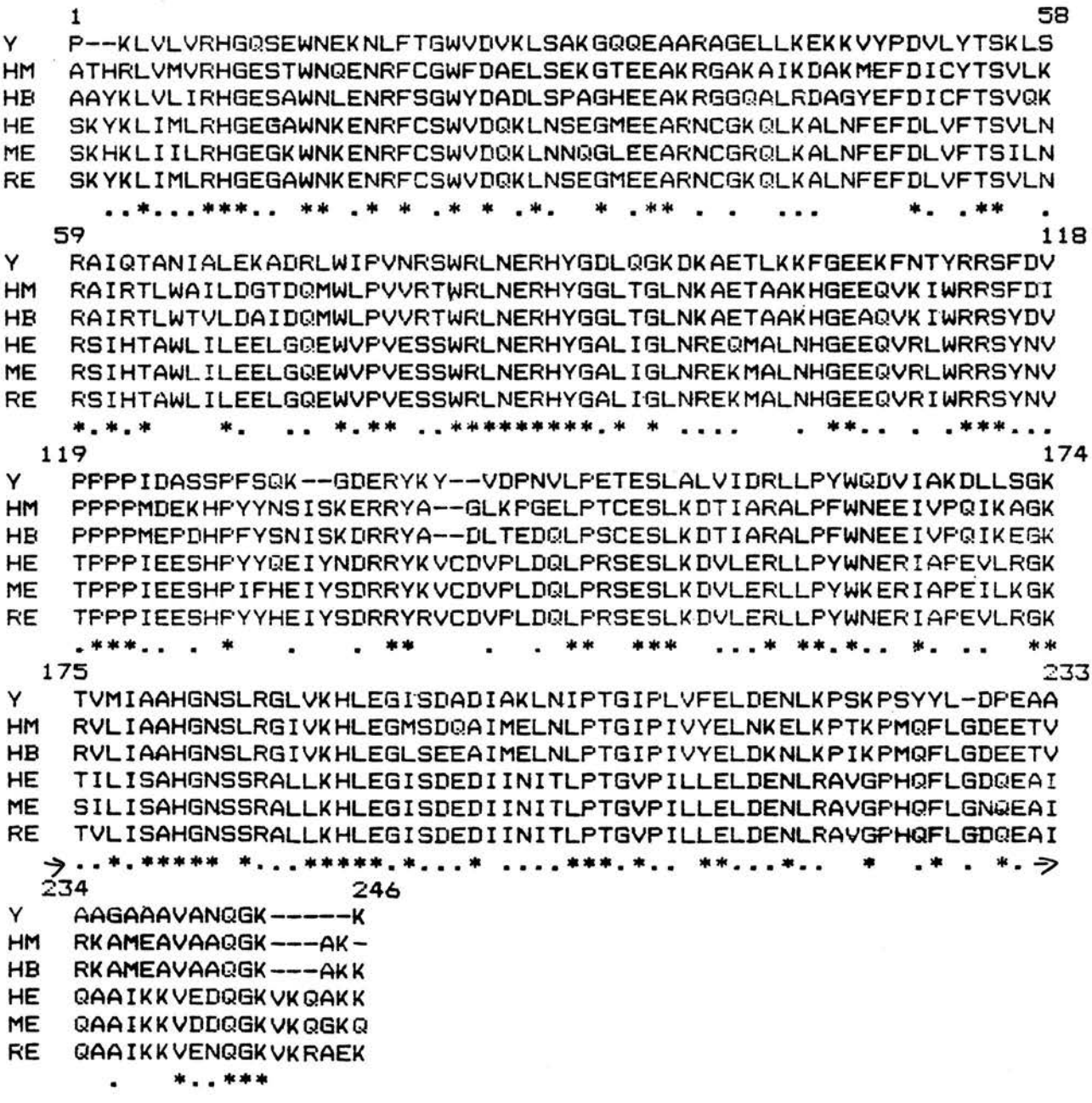

Figure 1.3. Alignment of the six known amino acid sequences of phosphoglycerate mutases (Y- yeast; HM- human muscle; HB- human brain; HE- human erythrocyte; ME- mouse erythrocyte; RE- rabbit erythrocyte). Residues identical in all six sequences are indicated by an asterisk and partially conserved residues by a full stop. The numbering corresponds to the yeast sequence. The sequence alignment was generated by the multi-alignment progam CLUSTAL developed by Higgins and Sharp (1988).

exact 222 symmetry (fig. 1.4). The polypeptide backbone folds into a single domain consisting of a core of ß-sheets surrounded by α-helices; a structural motif common to many dehydrogenases and kinases (Campbell et al., 1974). The active site, located by soaking crystals in the substrate 3-phosphoglycerate, is formed entirely by the residues of one subunit (figures 1.5 & 1.6). The unligated, native form of the enzyme has two sulphate ions at the active site, and their positions are presumed to correspond to those of the phospho-groups of the bound ligands. The C-terminal 'tail' of the enzyme, as deduced from the protein sequence, does not contribute to the electron density map, and is therefore considered to be flexible under the conditions in which the enzyme was crystallised.

1.8.3 The active site

The active site of the enzyme is shown in fig. 1.6. Histidine 8, which has been shown to become phosphorylated during the catalytic cycle, is present in a position close to the first sulphate ion (S1). This probably represents the position of the transferrable phospho-group of bound 2,3- or 1,3-bisphosphoglycerate. Parallel to His-8, and only 0.4nm distant, lies His-181. This residue is in a suitable position to act as a proton donor/acceptor during phospho-transfer. Both histidines are conserved in all mutases sequenced so far. The transferrable phospho-group would be in a suitable position to form hydrogen bonds with Ser-11 and Thr-20 in the yeast enzyme, and the expected third phospho-ligand may be provided by one of the two C-terminal lysine residues (see section

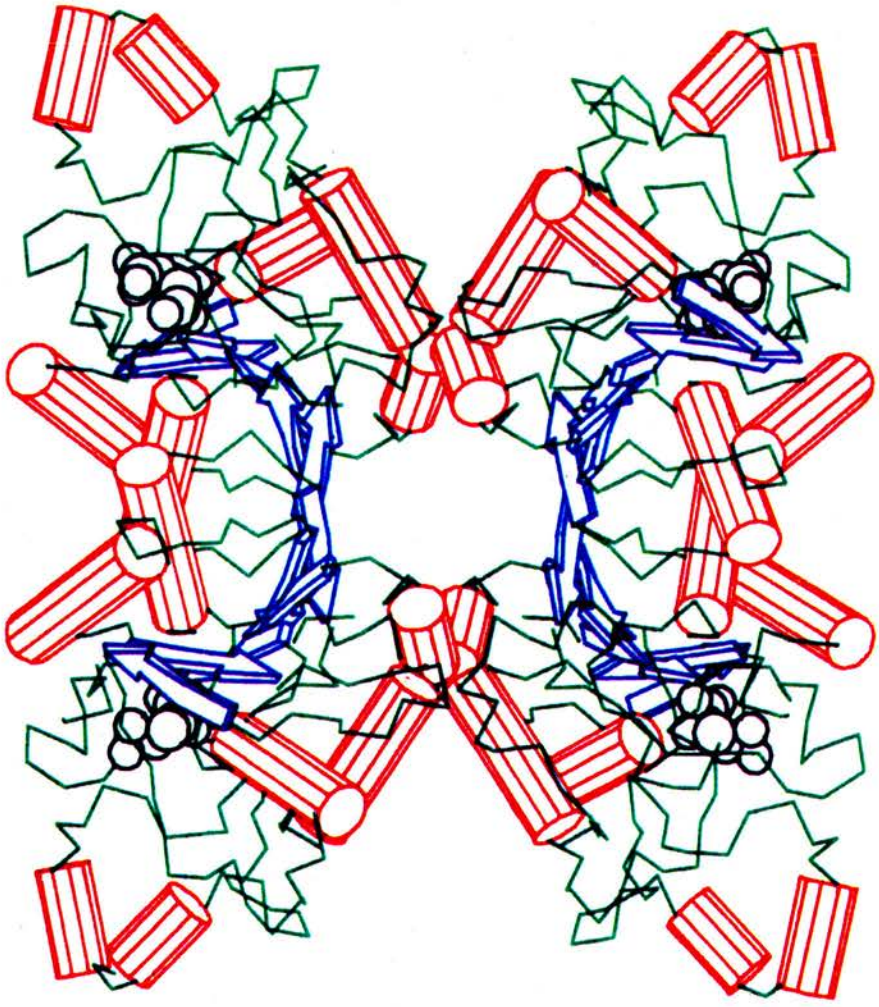

Figure 1.4. The structure of the yeast phosphoglycerate mutase tetramer. The α-helices are indicated by the red cylinders, and the ß-strands by the blue arrows. Green arrows correspond to portions of the polypeptide chain conecting elements of secondary structure. The four active sites are shown by space-filling models of 3-phosphoglycerate as located by crystal soaking experiments. This drawing and figure 1.5 appear courtesy of J. Littlechild and H. Watson of Bristol University.

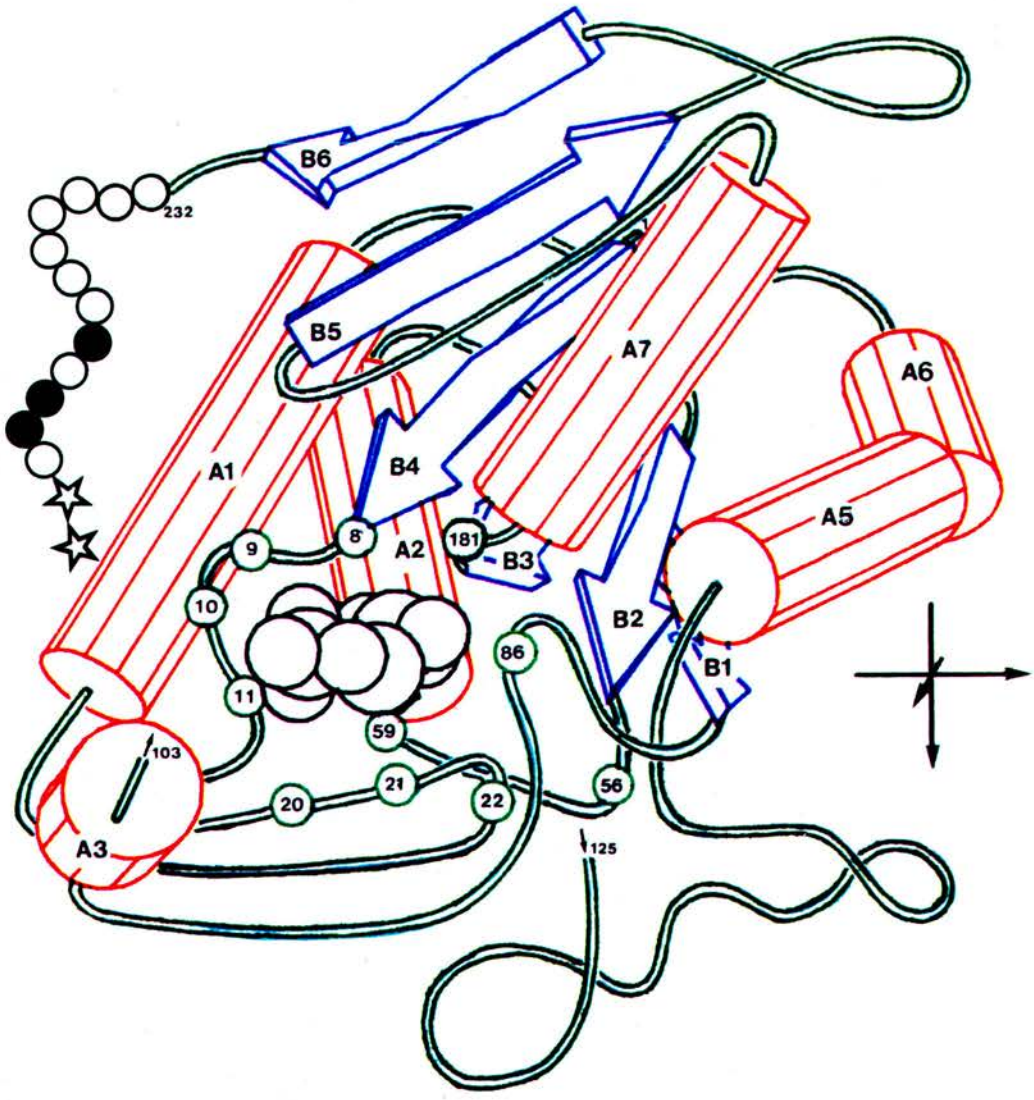

Figure 1.5. The yeast phosphoglycerate mutase subunit. The elements of secondary structure and the active site are indicated as for figure 1.4. The α-helices are labelled A1-A3 and A5-A7, and the ß-strands are labelled B1-B6. A portion of the polypeptide chain including helix A4 has been removed to reveal the active site. The α-carbon positions of the residues bordering the active site are indicated by numbered circles. The C-terminal tail has been represented by the following symbols - open circles represent ala and gly residues, closed circles represent residues with larger side chains and stars represent the two C-terminal lysine residues.

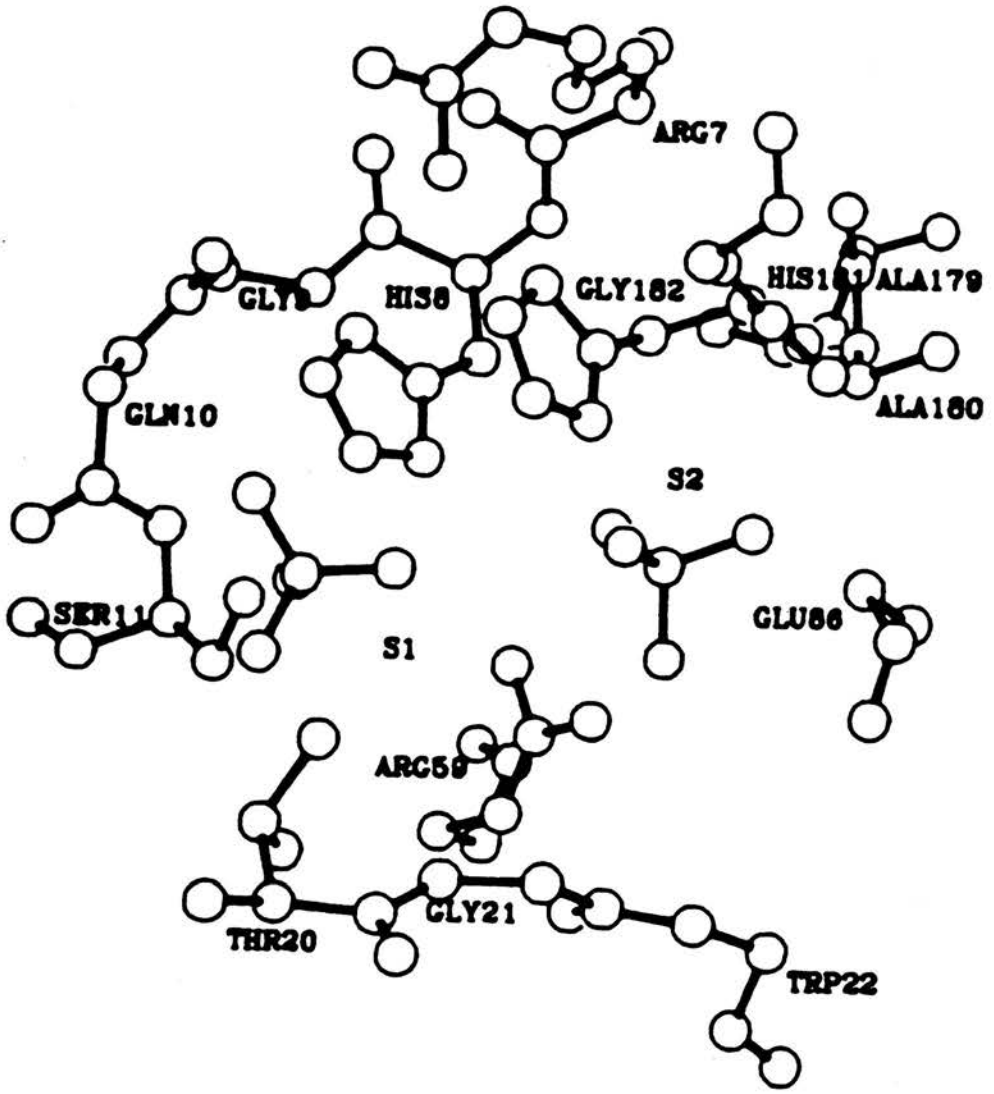

Figure 1.6. The active site of yeast phosphoglycerate mutase. The side-chains bordering the active site are labelled. The two sulphate ions found at the active site of the native, unliganded form of the crystal are drawn and are labelled S1 and S2. This diagram is reproduced from (Fothergill-Gilmore and Watson, 1989) with the permission of the authors.

1.11). The second, non-transferrable phospho-group (position S2) probably interacts with the positive dipole associated with the amino-terminus of helix 7. Arg-59, which is also conserved in all mutases, is in a suitable position to form a salt bridge with the carboxyl group of the bound ligands.

Yeast phosphoglycerate mutase has a high affinity for the cofactor 2,3-bisphosphoglycerate; the Km has been estimated as 0.8μM (Chiba et al., 1970). Model-building has shown that 2,3-bisphosphoglycerate can bind at the active site in either of two orientations, with the positions of the 2- and 3- phospho-groups reversed (fig. 1.7a; Fothergill-Gilmore and Watson, 1989). The implications of this observation for the reaction mechanism of the enzyme will be discussed in the following section. As shown in figures 1.7b and c, 3-phosphoglycerate and 2-phosphoglycerate probably bind to the phospho-enzyme with their phospho-group occupying the position of the second sulphate ion (S2) in the crystal structure. The enzyme must be flexible enough to accommodate the binding of each of these species. The Michaelis constants of yeast phosphoglycerate mutase for 2- and 3-phosphoglycerate have been estimated as <100μM and 710μM respectively (Rodwell et al., 1957; McAleese et al., 1987). The lower Km observed for 2-phosphoglycerate indicates that it can be more tightly bound than 3-phosphoglycerate.

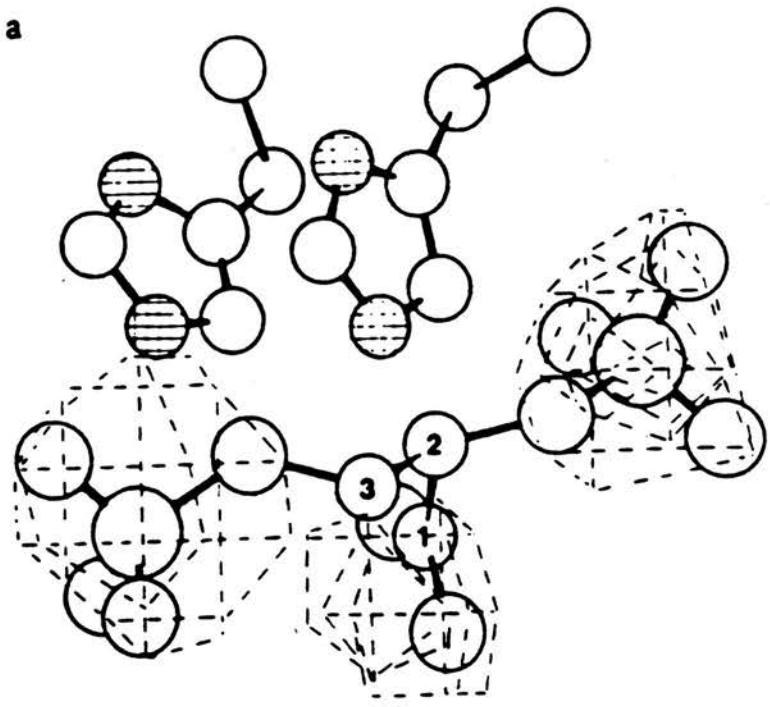

Figure 1.7. The proposed binding of cofactor and substrates at the active site of phosphoglycerate mutase. The positions of His-8 and His-181 are indicated, with the hatched atoms corresponding to the ring nitrogens. (a) 2,3-bisphosphoglycerate is shown bound with the 3-phospho group occupying the S1 or 'His-8' position and the 2-phospho group in the S2 or 'helix dipole' position. The carboxyl group is in a suitable position to interact with Arg-59. The dashed lines indicate electron density corresponding to ions bound at the active site of the unliganded enzyme. (b) 2-phosphoglycerate is shown bound at the active site of the catalytically-competent phosphoenzyme. The filled-in atom represents the proton that is abstracted or donated by His-181 during catalysis. (c) 3-phosphoglycerate shown in its bound position at the active site. The small differences in the positions of the carboxyl groups of 2-phosphoglycerate and 3-phosphoglycerate may account for the different affinities with which they are bound by the enzyme. This figure is reproduced from (Fothergill-Gilmore and Watson, 1989) with the permission of the authors.

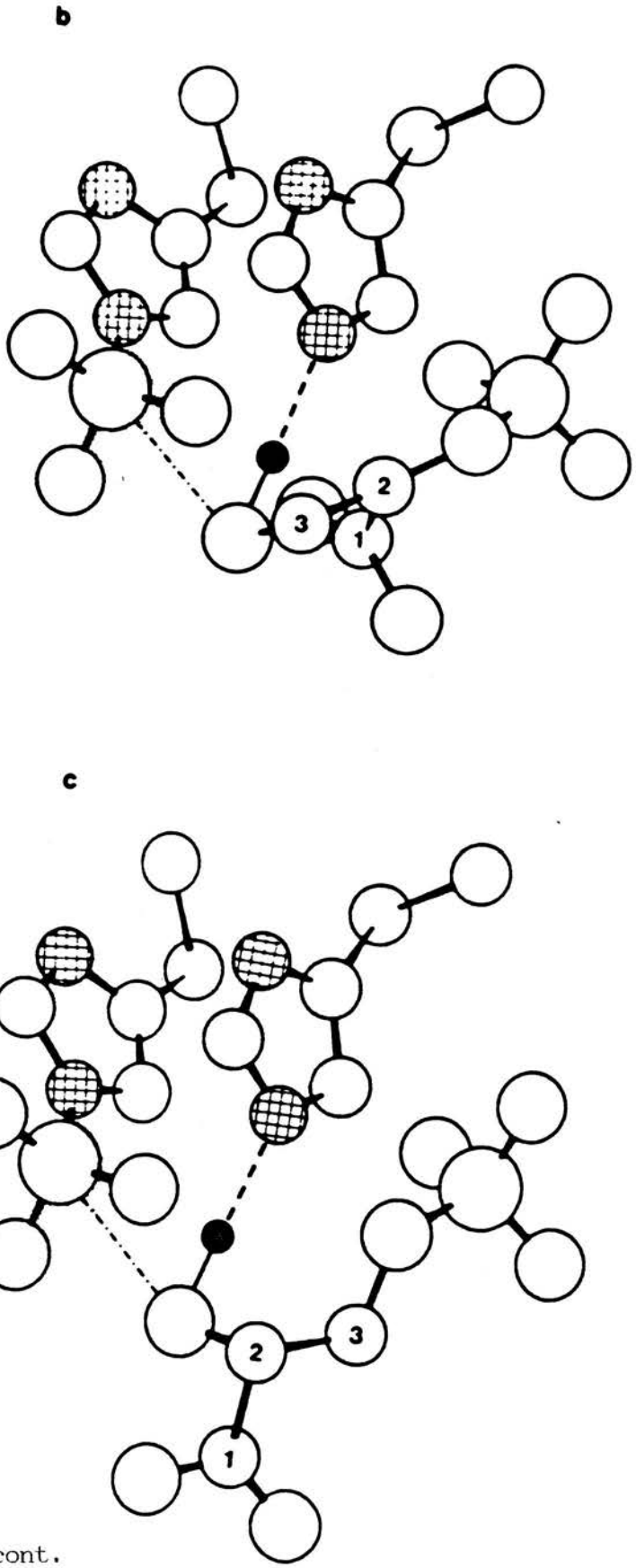

Figure 1.7. cont.

1.9 Proposed mechanism for phosphoglycerate mutases

Based on the extensive structural, kinetic and chemical analyses of phosphoglycerate mutases, a common reaction mechanism has been proposed, which can accommodate the mutase, synthase and phosphatase activities observed for the enzymes (Fothergill-Gilmore and Watson, 1989). The proposed reaction sequences are summarised schematically in fig. 1.8. The residue numbering used here refers to the yeast enzyme, the structure of which is best characterised.

1.9.1 Mutase reaction

The unphosphorylated form of the enzyme is labelled 'i' in fig. 1.8. Priming of the mutase reaction would occur when 2,3-bisphosphoglycerate bound to the unphosphorylated enzyme, in either of two orientations. In 'd' the 2-phospho group is in the transferrable position close to His-8, and probably forms hydrogen bonds with Ser-11, Thr-20 and one of the C-terminal lysines. The 3-phospho group is interacting with the positive charge associated with the dipole at the amino terminus of helix 7, and the carboxyl group forms a salt-bridge with Arg-59. In 'e' the positions of the phospho-groups are reversed.

Phospho-transfer to His-8 could then occur, with the donation of a proton by His-181, giving rise to 'c' and 'f' in fig. 1.8. The 2-phosphoglycerate or 3-phosphoglycerate formed would then be released, giving rise to the phosphorylated, catalytically competent form of the enzyme ('h' and 'g' in fig. 1.8). A round of

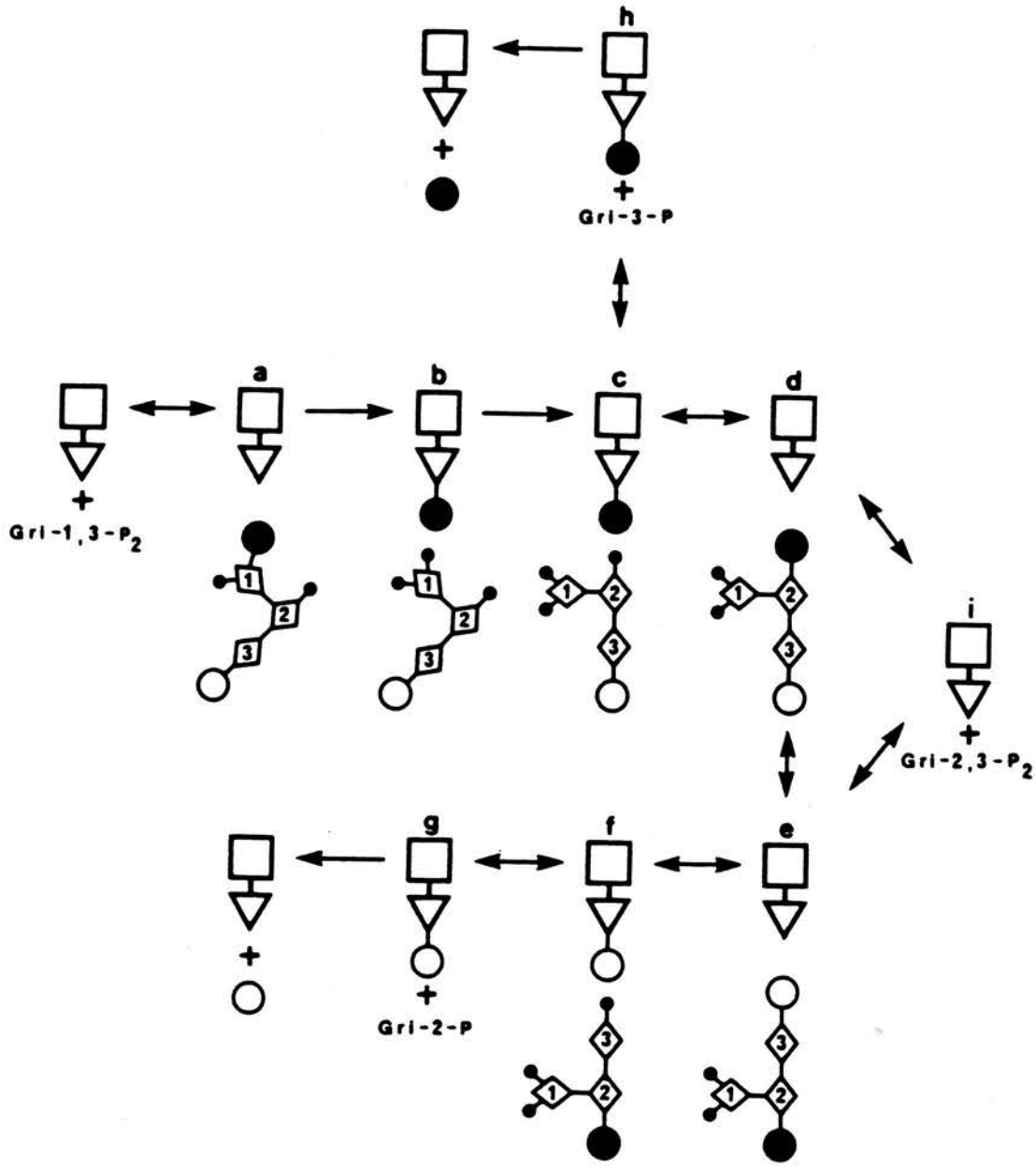

Figure 1.8. Proposed reaction sequence of cofactor-dependent phosphoglycerate mutases. The upper part of each diagram represents the active site of the enzyme with the triangle correponding to His-8. The numerals 1, 2 and 3 label the carbon atoms of glycerate. The large circles represent phospho-groups, and the small circles oxygen atoms of hydroxyl or carboxyl groups. Symbols (IUPAC-IUB Commission on Biochemical Nomenclature, 1978): Gri-2,3-P_2, 2,3-bisphosphoglycerate; Gri-1,3-P_2, 1,3-bisphosphoglycerate; Gri-2-P, 2-phosphoglycerate; Gri-3-P, 3-phosphoglycerate. This figure is reproduced from (Fothergill-Gilmore and Watson, 1989) with the permission of the authors.

catalysis would begin when either 3-phosphoglycerate (for glycolysis) or 2-phosphoglycerate (for gluconeogenesis) bound to the active site of the phospho-enzyme. Phospho-transfer from the enzyme to the substrate would produce 2,3-bisphosphoglycerate at the active site ('d' and 'e' in fig. 1.8). The proposed mechanism then requires that the cofactor re-orientates in the active site in order to allow the transfer of the phospho-group introduced by the substrate to the enzyme (the interconversion of 'd' and 'e' in fig. 1.8). Modelling studies have indicated that the size and shape of the active site pocket are such as to make this possible, assuming that the 2,3-bisphosphoglycerate could move away slightly from His-8 and His-181. During this reorientation, the cofactor could be retained in the active site by charge attraction and by the action of the C-terminal tail (Fothergill-Gilmore and Watson, 1989). Once reorientation had taken place, phospho-transfer would result in the formation of a mono-phosphorylated product, which could be released, and a phospho-enzyme, which could take part in another round of catalysis ('h' and 'g' in fig. 1.8).

1.9.2 Phosphatase reaction

The low phosphatase activity observed for phosphoglycerate mutases probably arises from the occasional hydrolysis of the phospho-enzyme, which has been calculated as having a half-life of 1-2 min (Britton et al., 1972b). The existence of a low rate of dephosphorylation of the enzyme gives rise to a requirement for a phospho-donor; in other words it is responsible for the 'cofactor dependence' of the enzyme. Phosphatase activity can be stimulated

by many anions, and particularly by the two-carbon substrate analogue 2-phosphoglycollate. It has been suggested that this analogue is sufficiently similar to the substrates to bind at the active site along with a molecule of water, enticing the C-terminal tail into the active site and promoting the transfer of the enzyme-bound phospho-group to the water molecule (Fothergill-Gilmore and Watson, 1989). In support of this theory, it has been observed that the 'tail-less' form of yeast phosphoglycerate mutase retains a basal phosphatase activity which can no longer be stimulated by 2-phosphoglycollate (Sasaki et al., 1971; see section 1.11).

1.9.3 Synthase reaction

The 2,3-bisphosphoglycerate synthase activity of phosphoglycerate mutase would be initiated when the substrate 1,3-bisphosphoglycerate bound to the active site of the unphosphorylated enzyme ('a' in fig. 1.8). It is likely that the substrate would bind so that the phospho-group on carbon 1 was in a suitable position for transfer to the enzyme, and that the 3-phospho group was in the helix dipole position. In this orientation Arg-59 would not be involved in binding the substrate. Once phospho-transfer had occurred ('b' in fig. 1.8), the negatively charged carboxyl group of the bound 3-phosphoglycerate would be repelled by the phospho group on His-8, and would probably move to form a salt bridge with Arg-59 ('c' in fig. 1.8). The 3-phosphoglycerate at the active site would now be in a suitable position to become phosphorylated at its carbon 2 position, in an identical reaction to that which occurs in the glycolytic mutase

reaction, forming 2,3-bisphosphoglycerate at the active site ('d' in fig. 1.8). Dissociation of 2,3-bisphosphoglycerate would give rise to the synthase activity, leaving the unphosphorylated enzyme ready to bind another molecule of 1,3-bisphosphoglycerate.

The reaction mechanism proposed above is consistent with the structural and kinetic data available for phosphoglycerate mutases. Furthermore, it serves to highlight the close relationship of the mutase and synthase activities, which only differ in the retention or release of the 2,3-bisphosphoglycerate which is formed in each reaction. This relationship, and a possible molecular basis for the differing ratio of the two activities found in the B/M-type and E-type isoenzymes, are discussed more fully in the following section.

1.10 The synthase/mutase relationship

As we have seen, the mutase and synthase activities of phosphoglycerate mutase are closely related - differing only in a tendency to retain (mutase) or release (synthase) 2,3-BPGA when it is formed at the active site. The ancestral form of the enzyme (corresponding to the M and B isoenzymes in vertebrates and the yeast enzyme) is primarily a constituent of the glycolytic/gluconeogenic enzyme pathway, and possesses a very high mutase:synthase activity ratio; typically 10000:1 in vertebrates such as pigs (Pons et al., 1985; Bartrons and Carreras, 1982) and 30000:1 in yeast (Sasaki et al., 1976). This suggests that 2,3-BPGA is very seldom released from the active sites of these enzymes. Occasionally, hydrolysis of the phospho-enzyme will occur, giving rise to a need for rephosphorylation. In vertebrate tissues, 2,3-BPGA exists at suitable levels to act as a phospho-donor (Tauler et al., 1987). In other organisms such as yeast, which possess no separate synthase isoenzyme, the levels of 2,3-BPGA may be extremely low. These enzymes may be re-phosphorylated by 1,3-BPGA, which is also known to act as a phospho-donor (Laforet et al., 1974), in which case they may be de facto 1,3-BPGA-dependent rather than 2,3-BPGA-dependent.

Somewhere early in vertebrate evolution a gene duplication event allowed the divergence of a new isoenzyme (E, also known as the 'synthase' enzyme) with an increased synthase activity. 1,3-BPGA

became an important substrate, and 2,3-BPGA a product rather than a transitory intermediate. What evidence exists about the relationship of the B/M and E isoenzymes?

1.10.1 Kinetic constants

Table 1.2. Comparison of mutase and synthase activities for the E isoenzyme with those of the B isoenzyme from three species of vertebrate.

species	mutase activity	synthase activity	reference
human	1.4%	45x	Ikura et al., 1978
chicken	0.08%	30x	Bosch et al., 1982
pig	0.3%	75x	Tauler & Carreras, 1987

Table 1.2 summarises the changes in mutase and synthase activities (compared to their B isoenzymes) found for the E isoenzymes of three species of vertebrates. On average, the E isoenzyme retains about 0.6% of the mutase activity of the B isoenzyme, and displays a fifty-fold increase in synthase activity. This shifts the mutase:synthase activity ratio from 10000:1 for the B form to about 1:1 for the E form. This dramatic change in the catalytic emphasis of the E isoenzyme is reflected in changes in the affinity constants for 2,3- and 1,3-BPGA. Thus the affinity for 2,3-BPGA appears to have dropped [Km 0.5μM for the chicken B and pig M isoenzymes (Rose and Dube, 1978; Bartrons and Carreras, 1982) to

40μM for the human E isoenzyme (Rose, 1980)] whilst the affinity for 1,3-BPGA has increased [Km 5μM for the rabbit M isoenyzme (Laforet et al., 1974) compared to 0.4μM for the human E isoenzyme (Pons and Carreras, 1986)]. This represents a change in the affinity ratio of 2,3-BPGA:1,3-BPGA by a factor of 100, from about 10:1 for the M isoenzyme to about 1:10 for the E isoenzyme.

1.10.2 Rates of phospho-transfer

Using a continuous flow and quench technique which allowed measurements of reactions as fast as 20msec, Rose and Dube (1976) investigated the rate of phosphorylation of the chicken B and horse E isoenzymes by 1,3- and 2,3-BPGA. These experiments were designed to determine whether or not a mechanism involving a phospho-enzyme intermediate was plausible, but they also highlighted some interesting distinctions between the B and E isoenzymes, as summarised in table 1.3.

Table 1.3. Rate of phosphorylation of B and E isoenzymes by 1,3- and 2,3-bisphosphoglycerate.

enzyme	rate of phosphorylation (s^{-1})	
	1,3-BPGA	2,3-BPGA
horse E	13.5	2.3
chicken B	1.57	>1000

The phosphorylation of the B isoenzyme by 2,3-BPGA was too fast to measure (but must have been at least as high as the k_{cat} for the mutase reaction, about $1100s^{-1}$), whereas phosphorylation by 1,3-BPGA was very much slower, limiting the rate of the synthase activity. In contrast 1,3-BPGA was found to be a better phospho-donor than 2,3-BPGA for the horse E isoenzyme, reflecting its increased synthase:mutase activity ratio. These changes are consistent with the observed changes in the Km's for the two moieties.

1.10.3 Molecular basis

What molecular events heralded the creation of an enzyme that preferred to release 2,3-BPG rather than recycle it? What alterations in the enzyme structure were responsible for the altered specificities described above? The recent publication of three mutase and three synthase (E) sequences, together with the solution of the yeast mutase crystal structure, allow us to make some educated guesses. The six sequences are shown aligned in figure 1.9. Overall they share about 35% sequence identity. If one restricts comparisons to the regions which appear to bound the active site in the yeast enzyme (boxed), then the identity rises to 61%. The active site of the E isoenzyme has been modelled to fit the known crystal structure of the yeast enzyme using the program FRODO, and the two active sites are shown in figures 1.10 and 1.11. There are only three positions in which one side-chain is conserved in mutases and another in synthases, possibly indicating important changes related to enzyme function. Firstly, there is position 11

(yeast numbering) which is a serine in mutases and is thought to provide a phospho-ligand for the transferrable phospho-group of 2,3-BPGA. In synthases, this residue is a glycine, and could thus perform no binding function. Secondly, there is residue 21 - a glycine in mutases and a serine in synthases. Inspection of the yeast structure reveals that the side-chain points away from the active site and therefore is unlikely to be involved in the binding of substrates. Lastly, there is residue 60 - an alanine in mutases but a serine in synthases. Intriguingly, this side-chain appears to be in a suitable position to provide a phospho-ligand for the non-transferrable phospho-group.

In other words, a 'shift' in phospho-ligands appears to have taken place in the synthase (E) enzyme with respect to its progenitor. Could this account for the observed reduction in the affinity for 2,3-BPGA and the concurrent increase in the affinity for 1,3-BPGA? As these substitutions presumably occurred successively, would each of the possible intermediate forms of the enzyme (ie S11/S59 and G11/A59) possess mutase and synthase activity, and in what ratios? These questions could be addressed by protein engineering.

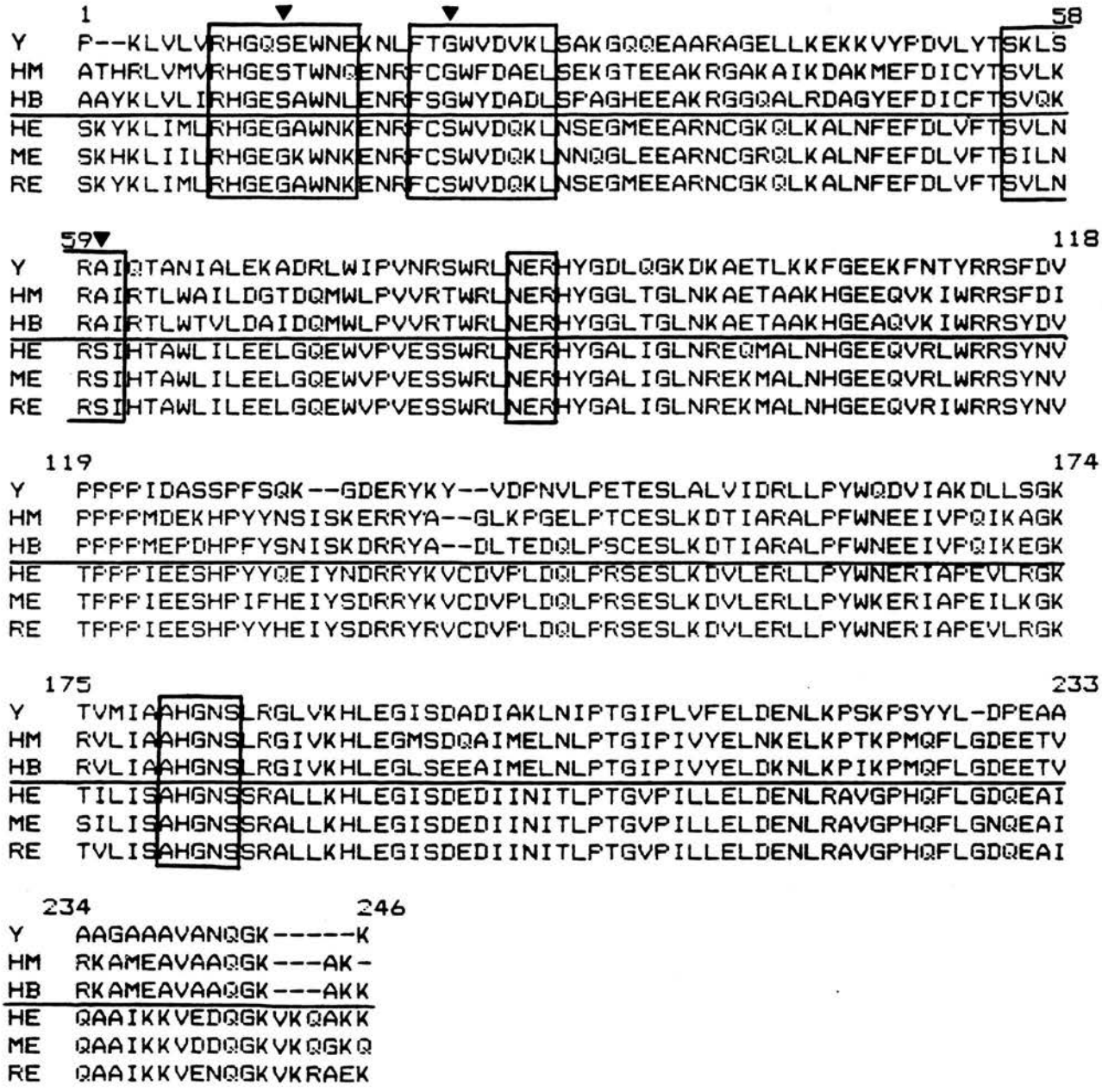

Figure 1.9. Alignment of the six known amino acid sequences of phosphoglycerate mutases. The sequences were aligned and labelled as for figure 1.3. Residues bounding the active site in the crystal structure of the yeast enzyme are boxed, and mutases are separated from synthases by a horizontal line. Residues marked with an arrow are discussed in the text.

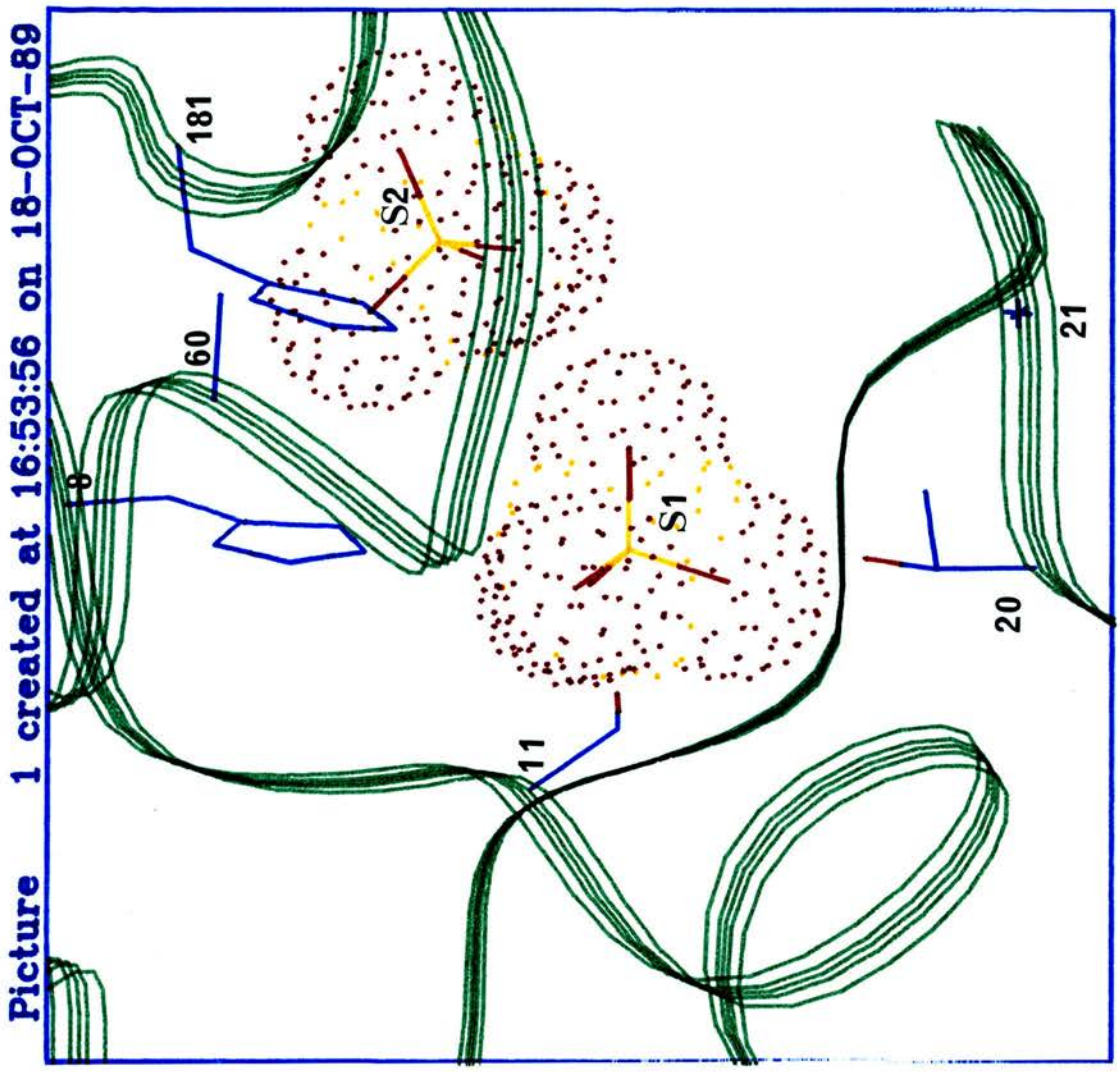

Figure 1.10. The active site of yeast phosphoglycerate mutase. The polypeptide backbone is represented by the green ribbon; side chains discussed in the text and the two sulphate groups are labelled. This diagram was produced using FRODO.

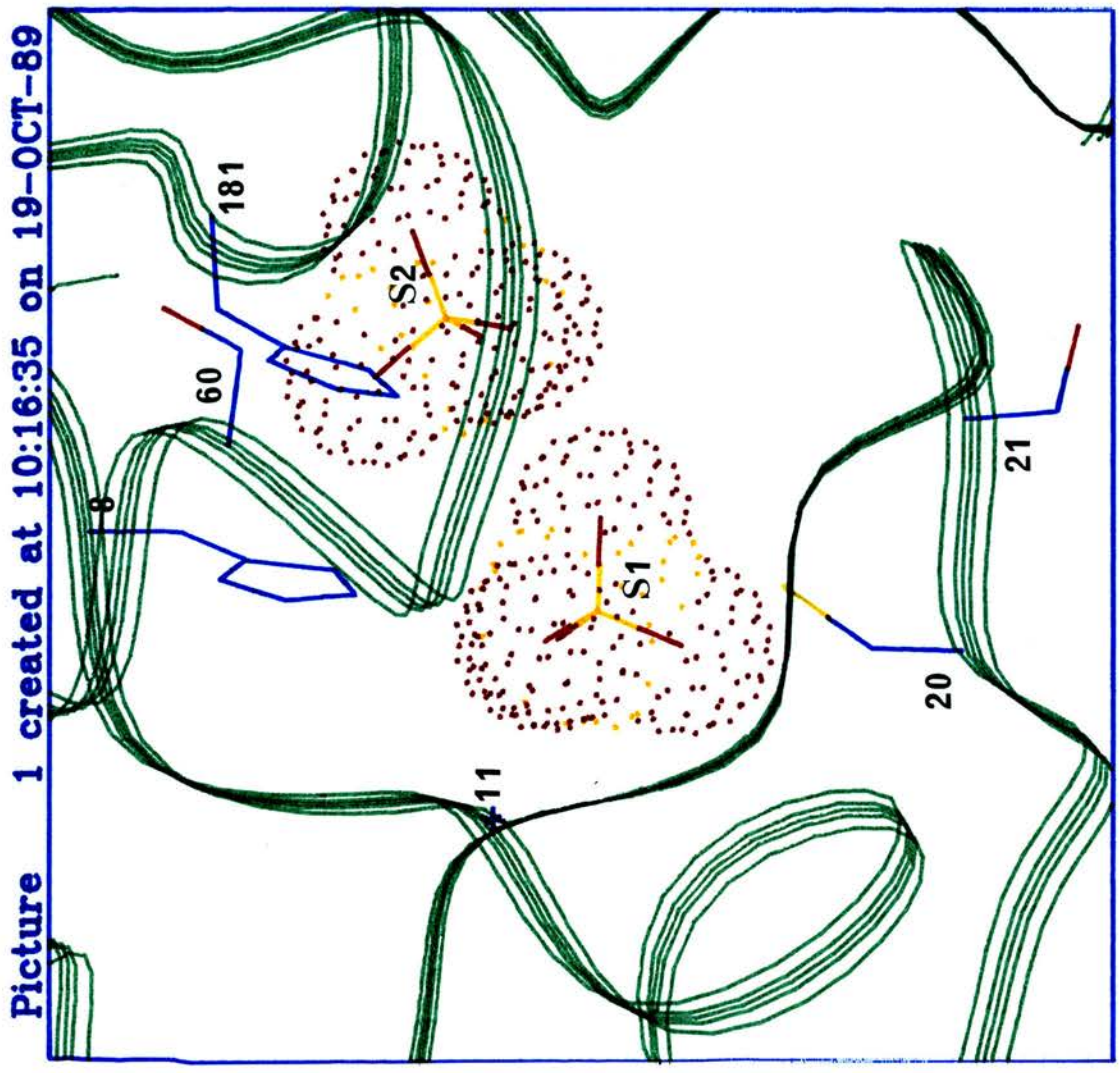

Figure 1.11. The modelled active site of a synthase enzyme. The polypeptide backbone is represented by the green ribbon; side chains discussed in the text and the two sulphate groups are labelled. This diagram was produced using FRODO.

1.11 The C-terminal tail

Early in studies on the purification of yeast phosphoglycerate mutase, it was noted that electrophoretically-separable components of differing activity were formed (Chiba and Sugimoto, 1959). Subsequently, five components were identified, from I - the most active, and probably wild-type, to V - the least active. Components II to V were progressively formed from component I during the autolysis of the yeast, and amino acid analysis of component V showed that it had lost about 12 residues of composition Glx, Asx, 2 Gly, 4.5 Ala, Val and 1.8 Lys when compared to component I (Sasaki et al., 1966). Analysis of the 2,3-BPGA phosphatase activity of components I and V showed that they possessed the same basal level of activity. However, when the potent phosphatase activator 2-phoshoglycollate was included, it was found that component V was much less strongly activated than component I (Sasaki et al., 1971b). The loss of about ten residues of rabbit muscle M-type phosphoglycerate mutase caused by proteolytic digestion with thermolysin resulted in the loss of all mutase activity but did not drastically change the conformation of the enzyme, which retained its ability to bind to cibacron blue-Sepharose (Price et al., 1985b).

The coincident solution of the crystal structure (Winn et al., 1981) and amino acid sequence (Fothergill and Harkins, 1982) of yeast phosphoglycerate mutase shed fresh light on these observations. It became apparent that the enzyme possessed a C-terminal 'tail' which did not crystallise, presumably due to its

flexibility (Winn et al., 1981). The amino acid sequence of this C-terminal region, with its high glycine and alanine content, supported the 'flexible tail' theory. It also gave a good match to the amino acid composition of the residues lost from component V. If a tail-less enzyme was inactive, then the tail must play an essential role in catalysis; but what might that be? Model building showed that the tail could modulate access to the active site of the enzyme (Winn et al., 1981), and it was suggested that the two C-terminal lysines might play some role in the active site itself (Fothergill and Harkins, 1982), possibly by providing an extra phospho-ligand for the transferrable phospho group. Lysine residues are found at the C-termini of the three mutases and the three synthases for which sequences are available (fig. 1.9).

The above evidence has led to the postulation of the following three roles for the C-terminal tail (Fothergill-Gilmore and Watson, 1989) :

i) It physically excludes water from the active site.

ii) It provides a ligand or ligands to stabilise the developing transition state, preventing phosphatase activity.

iii) It helps, by the charge stabilization of the lysine residues, to retain the cofactor/intermediate 2,3-BPGA during the reaction sequence.

Site-directed mutagenesis could be employed to test these ideas, for example by altering the length or flexibility of the tail or by changing one or both of the two C-terminal lysine residues.

1.12 Aim of project

The aim of this project was to improve our understanding of the molecular basis for the reactions catalysed by yeast phosphoglycerate mutase through the design and analysis of specific mutant forms of the enzyme. Recent rapid advances in recombinant DNA technology, and particularly in the technique of site-directed mutagenesis, have made such an approach feasible. The evidence gained from previous kinetic and structural studies of the enzyme, and detailed in the preceding pages, suggested two areas especially amenable to further study :

a) The importance of serine 11 to the mutase and synthase activities of the enzyme.

b) The role played by the C-terminal lysine residue in the three reactions catalysed by phosphoglycerate mutase.

1.13 Strategy of project

The ab initio requirement of any project involving site-directed mutagenesis is that the gene coding for the enzyme to be studied has been identified. In the case of yeast phosphoglycerate mutase, a recombinant vector isolated from a yeast chromosomal DNA library had been shown to complement a phosphoglycerate mutase deficient strain of yeast (Kawasaki and Fraenkel, 1982). This plasmid, which was presumed to carry the gene for yeast phosphoglycerate mutase, was kindly made available to us by Professor Dan Fraenkel.

The initial strategy of the project was to localise and sequence the phosphoglycerate mutase gene. A knowledge of the DNA sequence would allow the design of oligonucleotides which could introduce the desired mutations in the gene. In order to study the effect of the mutations generated, the mutant genes would be expressed in a strain of yeast which carried no chromosomal copy of the wild-type gene. The resultant mutant enzymes would be purified and kinetically characterised using established techniques in order to assess the effect of the mutations introduced.

2. Materials and Methods.

2.1 Materials

2.1.1 Strains

E.coli:

TG1 K12, Δ(lac-pro), supE, thi, hsdDS/F'traD36, proA$^+$B$^+$, lacI^q, lacZΔM15.

S.cerevisiae:

DBY747 leu2-3,2-112 his3A ura3-52 trp1.

DBYgpm- leu2-3,2-112 ura3-52 trp1 Δgpm.

2.1.2 Vectors

vector	source
YEP13.GPM	Professor D. Fraenkel, Harvard University.
M13mp19 M13mp9	Bethesda Research Laboratories (U.K.) Ltd., Cambridge, U.K.
pK19	Dr A. Boyd, Dept. of Biochemistry, University of Edinburgh.
YIP1	Dr J. Beggs, Dept. of Molecular Biology, University of Edinburgh.
pJDB207	Dr G. Reid, Dept. of Microbiology, University of Edinburgh.

2.1.3 Growth Media

a) Difco labs, Central Avenue, East Molesly, Surrey

Bactopeptone, purified agar, yeast nitrogen base without amino acids.

b) Oxoid Ltd., Haverhill, Suffolk

Agar No.1, tryptone, yeast extract.

c) Sigma Chemical Company, Poole, Dorset

Ampicillin, kanamycin, tetracycline, Xgal, IPTG, all amino acids and nucleotides.

2.1.4 Radiochemicals

Amersham plc, Lincoln Place, Aylesbury, Buckinghamshire

Deoxyadenosine 5'-(α-^{32}P)triphosphate, triethylammonium salt, stabilised aqueous solution 3000Ci/mmol.

Deoxyadenosine 5'-α-(^{35}S)thiotriphosphate, triethylammonium salt, stabilised aqueous solution >400Ci/mmol.

Adenosine 5'-(γ-^{32}P)triphosphate, triethylammonium salt, stabilised aqueous solution 3000Ci/mmol.

2.1.5 Enzyme assays and purification

a) Boeringher Mannheim, Bell lane, Lewes, East Sussex

Enolase, pyruvate kinase, lactate dehydrogenase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, creatine phosphokinase, creatine phosphate, 2-phosphoglycerate, 3,4-dichlorisocoumarin.

b) Sigma Chemical Company, Fancy Road, Poole, Dorset

3-phosphoglycerate grades I and II, 2,3-bisphosphoglycerate, NADH, AMP, ADP, ATP, 2-phosphoglycollate, Reactive blue 2-Sepharose CL-6B, E64c.

c) Aldrich Chemical Company Ltd., New Road, Gillingham, Dorset

1,10-phenanthroline.

2.1.6 Enzymes

a) Northumbria Biologicals Limited, South Nelson Ind. Est., Cramlington, Northumberland

Klenow fragment, T4 polynucleotide kinase, AluI, BamHI, BglII, EcoRI, HindIII, PstI, PvuII, SalI, SmaI, XhoI.

b) Boeringher Mannheim

T4 DNA ligase, RNase A, Proteinase K.

c) Sigma

Lyticase.

2.1.7 Oligonucleotides

All oligonucleotides were synthesised by:

OSWEL DNA service, Dept. of Chemistry, University of Edinburgh, West Mains Road, Edinburgh.

2.1.8 Miscellaneous

a) Amersham

Hybond-N roll, Hyperfilm MP X-ray film,
Site-directed mutagenesis kit.

b) Cambridge Bioscience, Newton House, Devonshire Road, Cambridge

Sequenase™ DNA sequencing kit.

c) Millipore (UK) Ltd.,Peterborough Road, Harrow, Middlesex

Millex-GS 0.22μm filter units.

d) Sigma

Polaroid 665 film, agarose, low melting temperature agarose

e) A & J Beveridge Ltd, 5 Bonnington Rd Lane, Edinburgh

5, 15 and 50ml Falcon tubes.

f) Pharmacia

Hexadeoxyribonucleotides.

All other biochemicals were supplied by Sigma and B.D.H., Poole, Dorset, and were reagent grade or better.

2.2 Methods

2.2.1 Experimental Precautions

Good laboratory practice was observed at all times. In work involving DNA manipulation or growth of micro-organisms, sterile technique and equipment were used. After use, plastic-ware was incinerated, and glassware used for the growth of bacteria or yeast was soaked in hypochlorite bleach prior to washing. The guidelines for the use of radioactive isotopes were strictly adhered to.

2.2.2 Standard experimental techniques

The standard experimental techniques listed below are described in *Methods in Enzymology 1988, **152** 'Guide to Molecular Cloning Techniques'* on the page numbers indicated.

Technique	page no.
Enzymic manipulation of DNA	91-144
Growth and storage of E.coli	145-151
Transformation of E.coli	151-154
Preparation of plasmids and phage	154-170
Preparation of yeast chromosomal DNA	493-494.

2.2.3 Radiolabelling of DNA by random priming

DNA probes from about two hundred to several thousand base pairs in length can be labelled to a very high specific activity by the technique of random priming (Feinberg and Vogelstein, 1983).

Solutions:

OLB	Solutions A, B and C in a ratio of 2:5:3 Store at -20°C; good for three months with repeated freezing and thawing.
Solution A	625µl 2M Tris-HCl pH 8.0 25µl 5M $MgCl_2$ 350µl SDW 19µl 2-mercaptoethanol 5µl 0.1M dCTP 5µl 0.1M dTTP 5µl 0.1M dGTP
Solution B	2M Hepes buffer titrated to pH 6.6 with NaOH
Solution C	Hexadeoxyribonucleotides evenly suspended in 3mM Tris-HCl, 0.2mM EDTA pH7.0 at 90 OD_{260} units/µl - stored at -20°C.
BSA	10mgml^{-1} enzyme grade
dATP	α-^{32}P-dATP, Amersham, 3000Ci/mmol, 10µCi/µl

Klenow fragment of DNA polymerase 1 diluted to 1.5 units/µl

Isolation of DNA for use as probe:

The DNA fragment desired as a probe was cut using the required restriction enzymes and separated in a low melting temperature agarose gel. The desired band was excised from the gel using a scalpel and placed in a pre-weighed microcentrifuge tube. SDW was added at a ratio of 1.5ml SDW to 1g of gel. The capped tube was placed in a boiling waterbath for 7 min and then allowed to equilibrate at 37°C for at least 10 min before labelling. Isolated fragments were stored at -20°C and reboiled for 3 min, equilibriated for 10 min prior to subsequent labelling. If it was unnecessary to isolate the probe from a gel, for instance if a whole plasmid was to be labelled, then the DNA was added directly to the labelling reaction below.

The labelling reaction:
reagents were added to a microcentrifuge tube in the following order:-

SDW	18µl
OLB	5µl
BSA	2µl
DNA	20µl (20ng)
dATP	3µl
Klenow	2µl

The reaction was allowed to run overnight at room temperature. The mixture was boiled for 5 min prior to hybridization.

2.2.4 Preparation and use of end-labelled oligonucleotide probes

Oligonucleotide probes can be used to detect a single base mismatch and are hence invaluable in distinguishing mutant from wild-type clones. They can also be designed from a protein sequence in order to detect the corresponding DNA sequence.

Solutions :

10x kinase buffer	1M Tris pH8.0
	100mM $MgCl_2$
	70mM DTT
	sterilize by filtration and store at -20°C.
20xSSC	3M NaCl
	0.3M trisodium citrate
Heparin	50 $mg.ml^{-1}$ in 4xSSC.

a) Preparation of labelled probe:
The following were mixed in a microcentrifuge tube:

Oligonucleotide (2.5-3 OD units/ml)	1.5µl
10x kinase buffer	3µl
30µCi [γ -^{32}P]ATP 3000Ci/mmol	3µl
SDW	22µl

2 units of polynucleotide kinase were added and the microcentrifuge tube was incubated in a lead pot at 37°C for 30 mins. The oligonucleotide was present in a 1.5 fold molar excess over the ATP, and it was not neccessary to remove unincorporated label. The labelled probe could be used immediately or stored at -20°C overnight.

b) Hybridization:

The Hybond-N filter (from Southern blot or slot-blot) was pre-hybridized in 10ml of (6xSSC, 0.2%(w/v) SDS, 40µl heparin) at 67°C for 1 hr in a suitable plastic box then removed and rinsed in 50ml of 6xSSC for 1 minute. The filter was then placed in a sealed plastic box with 10ml of 6xSSC; the labelled probe was added and hybridized at RT for 1 hour with gentle shaking.

c) Washing:

After hybridization, the filter was removed and washed in 100ml 6xSSC at RT for 5 minutes. At this temperature binding is fairly nonspecific. This was done three times, then the filter was wrapped in Saran-wrap and autoradiographed at -70°C for 1-4 hours or overnight. By increasing the washing temperature, an oligonucleotide can be made to differentiate between a sequence of perfect match and a sequence with one or more base mismatches. The temperature at which this occurs (Td) can be estimated by using the 'Wallace rule':

$$Td\ (^{\circ}C) = 4x(\text{no. of G \& C bases in oligonucleotide}) + 2x(\text{no. of A \& T bases in oligonucleotide})$$

The filter was rewashed at 5°C below the calculated melting temperature of the oligonucleotide in 6xSSC for 5 mins, then autoradiographed as before. If the probe was not distinguishing mutant from wild-type sequences then the temperature of the washing step was increased by 2°C.

2.2.5 Southern blotting

Solutions :

Denaturing buffer	1.5M NaCl 0.5M NaOH
Neutralizing buffer	1M Tris-HCl pH8.0 1.5M NaCl
20xSSC	see section 2.2.4.

Procedure:

After electrophoresis was completed, the agarose gel was transferred to a plastic tray and any unused areas of the gel were trimmed away with a scalpel. The gel was denatured by shaking gently in several volumes of denaturing solution for 1 hour at RT, then the denaturing solution was poured off and the gel was blotted dry with paper tissues, and covered with several volumes of neutralizing solution. After shaking at RT for at least one hour (up to 4 hrs is acceptable), the neutralizing solution was discarded and the blotting apparatus was prepared as follows. A sponge was placed in a plastic tray half-filled with transfer buffer (10xSSC). The sponge was covered with a piece of Whatman

3MM filter paper, pre-soaked in transfer buffer, which could dip into the buffer at either side, acting as a wick. The gel was placed on top of the wick and any trapped air bubbles were squeezed out. The gel was carefully surrounded with cling film to prevent 'short-circuits' between wick and paper towels. Next, a piece of Hybond-N cut to the exact size of the gel was placed on top of the gel, again avoiding air bubbles. Two sheets of pre-soaked 3MM paper were placed on top of the Hybond filter, followed by a 5cm stack of paper towels. Finally, a glass plate was placed on the top of the stack with a 1kg weight on top. The blot was left at RT overnight to allow DNA transfer to occur then the apparatus was dismantled and the filter was allowed to air dry for 1 hr and wrapped in Saran-wrap and placed DNA side down on a transilluminator for 3-5 mins to fix the DNA. The filter was now ready for hybridization.

2.2.6 Slot-blotting

Slot-blotting of denatured DNA samples onto Hybond-N filters was achieved using the Minifold II apparatus supplied from Schleicher and Schuell.

2.2.7 Heparin hybridization

This method of hybridization is very simple, and heparin provides a cost-effective means of controlling background hybridization. (Singh and Jones, 1984)

Solutions:

Formamide 100%, deionised.

Heparin see section 2.2.4.

Hybridization solution (HS)

2ml 20xSSC
500µl 2% Na pyrophosphate
5ml formamide
40µl heparin
460µl SDW
2ml 10% SDS (add last)
Total volume 10ml - may need more if >1 filter is to be probed.

Procedure:

HS was warmed to 42°C and the Hybond blot was pre-hybridised at 42°C for 10 min or longer, then placed in a sealable plastic bag. The HS was added and the bag was heat-sealed after expelling as much air as possible. The labelled probe was boiled (if double-stranded) for 5 min, then a corner was cut off the bag and the denatured probe was added to the HS. The bag was resealed and the contents were thoroughly mixed. The bag was placed in a waterbath at 42°C and shaken overnight. The next morning the bag was opened and HS disposed of carefully in a designated sink. The blot was carefully removed from the bag and washed in a plastic box at the

required temperature and ionic strength for 15 min. When probing for identical sequences, a stringent wash in 0.1xSSC at 65°C was required. The blot was checked for activity with a Geiger counter, and rewashed if necessary. Finally, the blot was wrapped whilst still moist in Saran-wrap and autoradiographed.

Re-use of filters:

If a Hybond filter was to be re-probed, hybridised probe was first stripped off. The filter was first incubated at 45°C in 0.4M NaOH, then transferred to 0.1xSSC, 0.1% (w/v) SDS, 0.2M Tris-HCl pH7.5 and incubated for a further 15 min. It could then be stored at 4°C in Saran-wrap until required for re-hybridisation.

2.2.8 DNA sequencing

The 'Sanger' or 'dideoxy' method of DNA sequencing was used, as supplied in kit form (Sequenase™ DNA sequencing kit, available from Cambridge Bioscience). The use of Sequenase™, a modified T7 DNA polymerase, allowed extended sequences of up to 500 bases to be read from a single primer. Sequencing gels were run on a BRL S0 sequencing system then fixed, dried down on 3MM paper and autoradiographed at RT overnight using Hyperfilm MP.

2.2.9 Site-directed mutagenesis

Several methods for oligonucleotide-directed in vitro mutagenesis have been developed (for review, see Carter, 1986). All require a single-stranded DNA template (usually phage M13) which carries the

gene to be mutated and an oligonucleotide carrying the desired mutation which can act as a primer for DNA synthesis. All give rise to a mixed dsDNA molecule composed of one mutant and one wild-type DNA strand. The methods differ in the way that they enrich for the mutant DNA sequence.

The method chosen here was that first reported by Fritz Eckstein and his co-workers (Taylor et al., 1985) and supplied in kit form by Amersham. The protocol is summarised in figure 2.1. Briefly, it makes use of the finding that dCTPαS, a phosphorothioate analogue of dCTP, can be incorporated into a growing DNA strand by DNA polymerase but will render that strand resistant to cutting by restriction enzymes such as NciI. Utilizing this protective effect, the wild-type DNA strand is nicked by NciI and subsequently removed by digestion with ExoIII, leaving the mutant strand as a template for the synthesis of wholly-mutant dsDNA. When transformed into E.coli, this DNA should give rise to a high percentage of mutant plaques.

2.2.10 Growth of yeast

Yeast can be grown in rich non-selective media, or in synthetic media which allows selection for those cells which have the ability to synthesise a particular amino acid such as leucine or histidine. Thus a strain such as DBY747 which is leu2- will only grow in leu- media when transformed with a plasmid carrying a functional LEU2 allele.

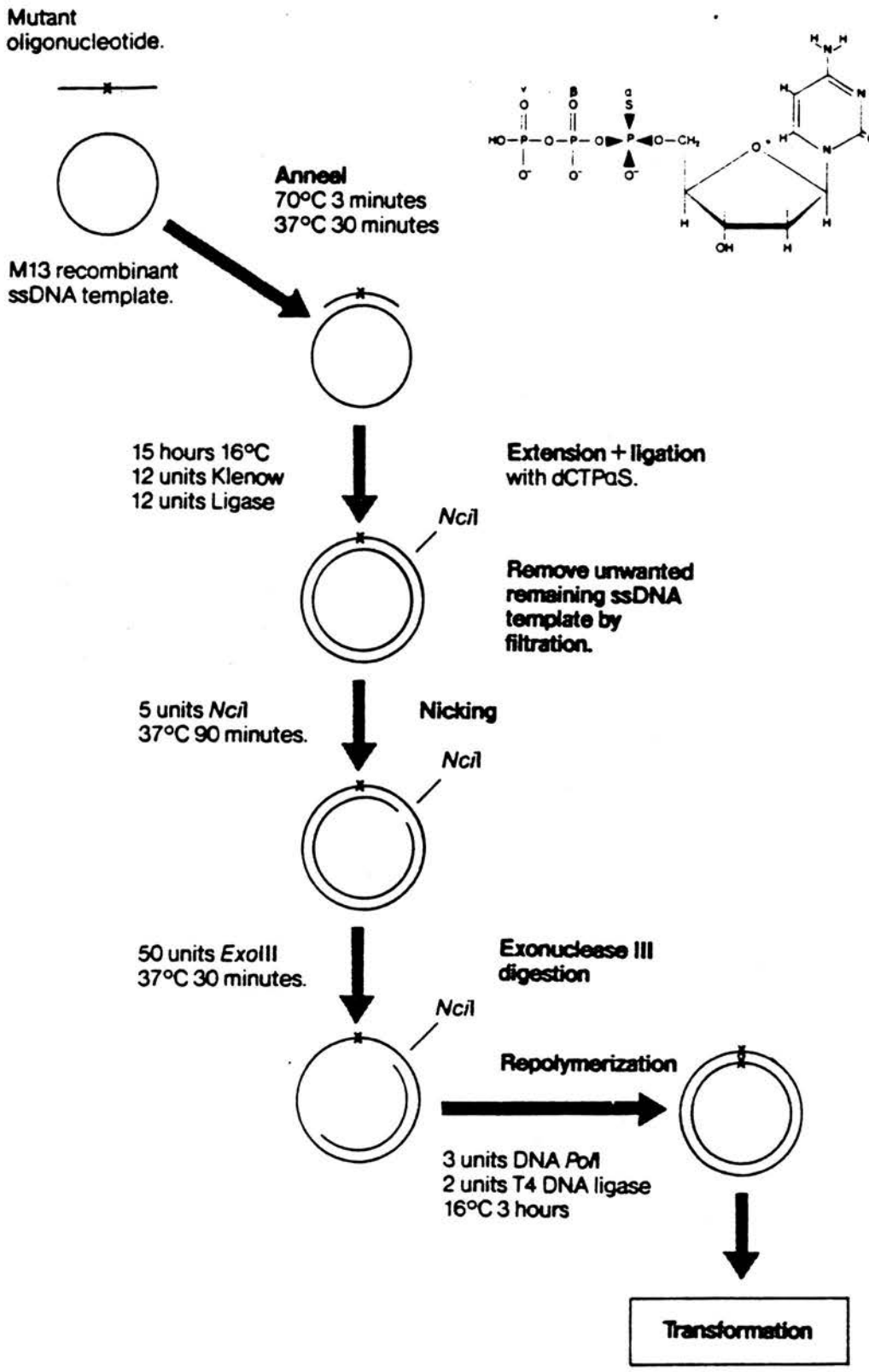

Figure 2.1. The Eckstein method of oligonucleotide-directed mutagenesis. The structure of the phosphorothioate analogue of dCTP is indicated in the top right hand corner (adapted from the Amersham handbook 'Oligonucleotide-directed in vitro mutagenesis system.').

Growth media

(1) Amino acid stock solutions

omission stock:

	g/100ml		g/100ml
Adenine	0.1	Isoleucine	1.5
Uracil	0.1	Phenylalanine	0.25
Tyrosine	0.12	Valine	0.75
Lysine	0.12	Threonine	1.0
Arginine	0.1	Serine	1.75
Methionine	0.1	Glutamic acid	0.5
Aspartic acid	0.5		

Leucine stock solution : 0.5% (w/v) leucine

Tryptophan stock solution: 0.2% (w/v) tryptophan

Histidine stock solution: 0.1% (w/v) histidine

Each stock solution was made up with distilled water, sterilised by autoclaving at 15lb/in^2 for 15 min and stored at 4°C (tryptophan stock was stored covered in aluminium foil to keep out light). To produce the desired selective media or plates, 10ml of each of the stock solutions required were added by sterile technique to 970ml of YOD/YOGE media or agar prior to pouring.

(2) Media

YEPD	Yeast extract	1% (w/v)
	Bactopeptone	2% (w/v)
	Glucose	2% (w/v)

YOD	Yeast nitrogen base (without amino acids)	0.67% (w/v)
	Glucose	2% (w/v)

The above media were made up to the required volume with distilled water and sterilised by autoclaving.
Glucose was replaced by 3% (w/v) glycerol plus 3% (w/v) ethanol in the recip es above to produce YEPGE and YOGE media respectively.

(3) Agar

YEPD/YEPGE agar: 20g agar No.1 made up to 1 litre with YEPD/YEPGE media and sterilised by autoclaving.

YOD/YOGE agar: 20g purified agar made up to 1 litre with YOD/YOGE media and sterilised by autoclaving.

Regeneration agar: 181.2g/l sorbitol added to the required agar prior to autoclaving.

2.2.11 Transformation of yeast

There are two distinct methods for the transformation of yeast - one which uses alkali cations such as lithium, and the other which involves the enzymatic digestion of the yeast cell wall.

2.2.11.1 Spheroplast transformation

Successful transformation of yeast spheroplasts was first reported by Beggs (1978). This method is more complex and requires more care than the LiAc method, but is capable of generating much higher

transformation efficiencies. The method used here is that of Burgers and Percival (1987). The enzyme used is lyticase, which is prepared from the gut of a snail!

Solutions:

SCEM 1M sorbitol
0.1M sodium citrate pH 5.8
10mM EDTA
autoclave then add 30mM 2-mercaptoethanol.

STC 1M sorbitol
10mM Tris.HCl pH 7.5
10mM $CaCl_2$

PEG 10mM Tris.HCl pH 7.5
10mM $CaCl_2$
20% PEG 8000
filter sterilize

SOS 1M sorbitol
6.5mM $CaCl_2$
0.25% yeast extract
0.5% bactopeptone
filter sterilize.

Transformation efficiency is very dependent on careful handling of the fragile spheroplasts. Whole cells were spun gently at 2000rpm (500*g*) and spheroplasts at 1200rpm (200*g*) in a MSE benchtop microcentrifuge at room temperature in order to maximize survival. A 50ml culture of the strain to be transformed was grown up overnight a density of about $3x10^7$ cells.ml^{-1}, transferred to a 50ml Falcon tube and harvested by centrifugation at 4°C for 10 min. The cells were washed successively in 20ml SDW and 20ml 1M sorbitol

by resuspension followed by 5 min spins, then resuspended in 20ml SCEM. 1000U of lyticase was added and the cells were incubated at 30°C with occasional inversions. Spheroplasting was monitored by measuring the decrease in absorbance at 800nm of a ten-fold dilution of cells in water. Spheroplasts lyse under these conditions, giving rise to a decrease in absorbance at 800nm. When over 90% were spheroplasts (15-20 min), cells were harvested for 3-4 min and the pellet was washed successively in 20ml 1M sorbitol and 20ml STC by gentle resuspension followed by 3-4 min spins. Finally, the pellet was resuspended in 2ml STC. Aliquots of 0.1ml were taken and mixed with plasmid DNA plus carrier (sonicated herring sperm) DNA to a total of 10µg in a 15ml Falcon tube and left at RT for 10 min. 1ml PEG was added and the tubes were inverted several times to mix, then incubated at RT for a further 10 min prior to harvesting for 4 min. The pellets were resuspended in 150µl SOS and incubated at 30°C for 20-30 min or overnight. To plate out, 8ml molten regeneration agar kept at 45-46°C was added mixed by inversion and poured onto warm selective plates. Once the agar had solidified, the plates were inverted and incubated at 30°C. Colonies appeared in 3-7 days.

2.2.11.2 Lithium acetate transformation

Transformation of intact yeast cells treated with alkali cations was reported by Ito et al., (1983). This method of transformation is quick and simple, but may not give a high enough transformation efficiency for some purposes.

Solutions:

LA 0.1M lithium acetate in TE (10mM Tris-HCl pH8.0, 1mM EDTA)

PEG 50% PEG 4000
filter sterilized

SOS Non-selective media with appropriate amino-acid supplements

Procedure:

A 50ml culture of the recipient strain was grown to a density of approx. $1x10^7$ ml^{-1}, harvested with a 5 min spin (500*g*, 2000rpm in an MSE benchtop centrifuge) and resuspended in 10ml TE, then spun down again and resuspended in 5ml LA buffer. The cells were shaken for 1 hr at 30°C, then dispensed into 0.3ml aliquots in microcentrifuge tubes. Plasmid DNA, plus carrier DNA to a total of 10µg, was added, followed by 0.7ml PEG. Tubes were inverted several times to mix and placed in a 30°C waterbath for 1 hour, then heatshocked for 5 min at 42°C. The cells were then centrifuged for 10s at low speed in a microcentrifuge, the supernatant discarded and the cells resuspended in 0.3ml SOS. Samples were incubated at 30°C for 20-30 min or overnight, then spread on to warm selective plates. Once dry, plates were inverted and incubated at 30°C. Colonies appeared in 2-4 days.

2.2.11.3 Analysis of yeast transformants

Yeast colonies obtained from selective plates must be analysed to ensure they carry the correct plasmid and are not due to

contamination. The most convenient way to do this is to rescue the plasmid from yeast and use it to retransform E.coli. Plasmid isolation from E.coli and restriction mapping can then be used to confirm the identity of the plasmid.

Procedure:

A 5ml culture of cells was grown in selective media overnight, transferred into microcentrifuge tubes and harvested by centrifugation at low speed in a microfuge then resuspended in 0.4ml SCEM. 50 units of lyticase were added and the cells were incubated at 30°C for 20 mins, checking for spheroplasts under the microscope (mixing 5µl cells with 5µl 10% SDS and looking for >90% cell lysis). Spheroplasts were spun down for 10s in a microfuge at low speed and gently resuspended in 0.45ml of TE. 50µl 10% SDS was added and mixed with a micropipette tip, then the tubes were incubated at 65°C for 30 min. 80µl 5M KAc was added, mixed, and left on ice for at least one hour, then the tubes were centrifuged for 15 min in a microfuge at high speed. The supernatant was transferred into a fresh tube (the pellet contained cell debris and was discarded). The DNA was precipitated by adding 1ml cold ethanol, mixing, then centrifuging for 5 min. The pellet was rinsed with 1ml cold 80% ethanol and air dried at 42°C, then resuspended in 0.5ml TE. 5ul RNase (10mg/ml) was added, mixed, and incubated at 37°C for 20 min. An equal volume of cold isopropanol was then added, mixed gently then centrifuged at high speed in a microfuge for 5 min. The pellet was rinsed and air dried as before, then resuspended in 20µl TE. Plasmids could sometimes be

visualised directly by running in an agarose gel, however it was usually necessary to transform E.coli with the DNA and undertake restriction analysis from bacterial minipreps.

2.2.12 Purification of yeast phosphoglycerate mutase

This method is suitable for the purification of 0.3-5mg of phosphoglycerate mutase from a yeast strain in which it is over-expressed.

2.2.12.1 Cell lysis and ammonium sulphate cut:

A 0.5-2 litre culture of yeast, from which phosphoglycerate mutase was to be purified, was grown to early stationary phase, transferred into 250ml centrifuge bottles and centrifuged at 6000rpm (5500*g*), 5 min, 4°C (JA14 rotor in J2-21 centrifuge). The supernatant was poured off, leaving a large cell pellet which was resuspended in 2-3 ml ice-cold 10mM Tris-HCl pH 7.5. The slurry was transferred into a small glass beaker (50-100ml) and the volume estimated. Protease inhibitors were added (see section 2.2.12.5), followed by glass beads (0.4mm diam.) until no surface liquid was visible. The beaker was placed in an ice-bucket and the cells were homogenised with a motorised teflon pestle at 1200rpm for 1.5-2 min (when slurry became more fluid and possibly frothy). 10-15ml cold 10mM Tris-HCl pH 7.0 was added and the beads were stirred with a glass rod to wash out protein, pouring the supernatant into a 100ml measuring cylinder. This step was repeated 4-5 times until the supernatant became almost clear. After estimation of the

volume it was transferred into a 250ml conical flask and enough solid ammonium sulphate to make a 55% saturated solution was added. The flask was stirred slowly on ice for 10-15 min to dissolve the salt, then heated in a water bath to 70°C for 5 min, stirring occasionally, to denature proteins, cooling to 4°C on ice afterwards. The liquid was then immediately transferred into SS34 centrifuge tubes and spun at 12000rpm (17400*g*), 25 min, 4°C (JA20 rotor in J2-21 centrifuge). After this step much of the protein and cell debris had precipitated, leaving most of the mutase in solution. The supernatant was decanted, its volume estimated and solid ammonium sulphate was added to make the solution 70% saturated. It was then stirred slowly on ice for at least 30 min (or kept overnight in the fridge). The solution was transferred into SS34 tubes and spun as before to precipitate the enzyme. The supernatant was discarded and the pellet resuspended in a small volume of 80% saturated ammonium sulphate (1-2ml) and transferred into a microcentrifuge tube. The enzyme is stable indefinitely in these conditions at 4°C.

2.2.12.2 Affinity chromatography:

As previously discussed, cofactor-dependent phosphoglycerate mutases from all sources studied share the ability to bind to cibacron-blue Sepharose. This property is shared by many glycolytic enzymes, probably refecting their common structural motif of α-helix and ß-sheet (Price and Stevens, 1983).

Phosphoglycerate mutase can be purified by allowing binding to cibacron blue-Sepharose and then specifcally eluting the enzyme with substrate or cofactor.

Procedure:

This procedure is based on that reported by Price and Stevens (1983) but uses 3-PGA for the specific elution of phosphoglycerate mutase rather than 2,3-BPGA, which was found to be less specific. The sample obtained in section 2.2.12.1 was centrifuged at high speed for 10 min in a microcentrifuge. The pellet was re-dissolved in 500µl 10mM Tris-HCl pH7.5 ('Tris') and dialysed overnight against 2l Tris at 4°C to remove the ammonium sulphate. A cibacron-blue Sepharose column (10cm by 1cm internal diameter) was equilibrated with Tris. The dialysed sample, about 1ml in volume, was added to the top of the column. The column was washed with Tris, pumped at one column volume per hour, until all the unbound material had been eluted. A UV monitor was used to detect protein in the eluent at 280nm. Next, two column volumes of Tris plus 1mM NADH, 1mM AMP were passed through the column to elute dehydrogenases and kinases, followed by two column volumes of Tris. Phosphoglycerate mutase was eluted by washing with two column volumes of Tris plus 10mM 3-PGA (Sigma grade II sodium salt). The enzyme normally eluted in a volume of 4-5ml. Finally, the column was washed with 2 column volumes of Tris plus 5M NaCl to elute all remaining bound proteins and to regenerate the column. A typical trace of absorbance at 280nm of eluted material is shown in figure 2.2. The eluted phosphoglycerate mutase was assayed for protein

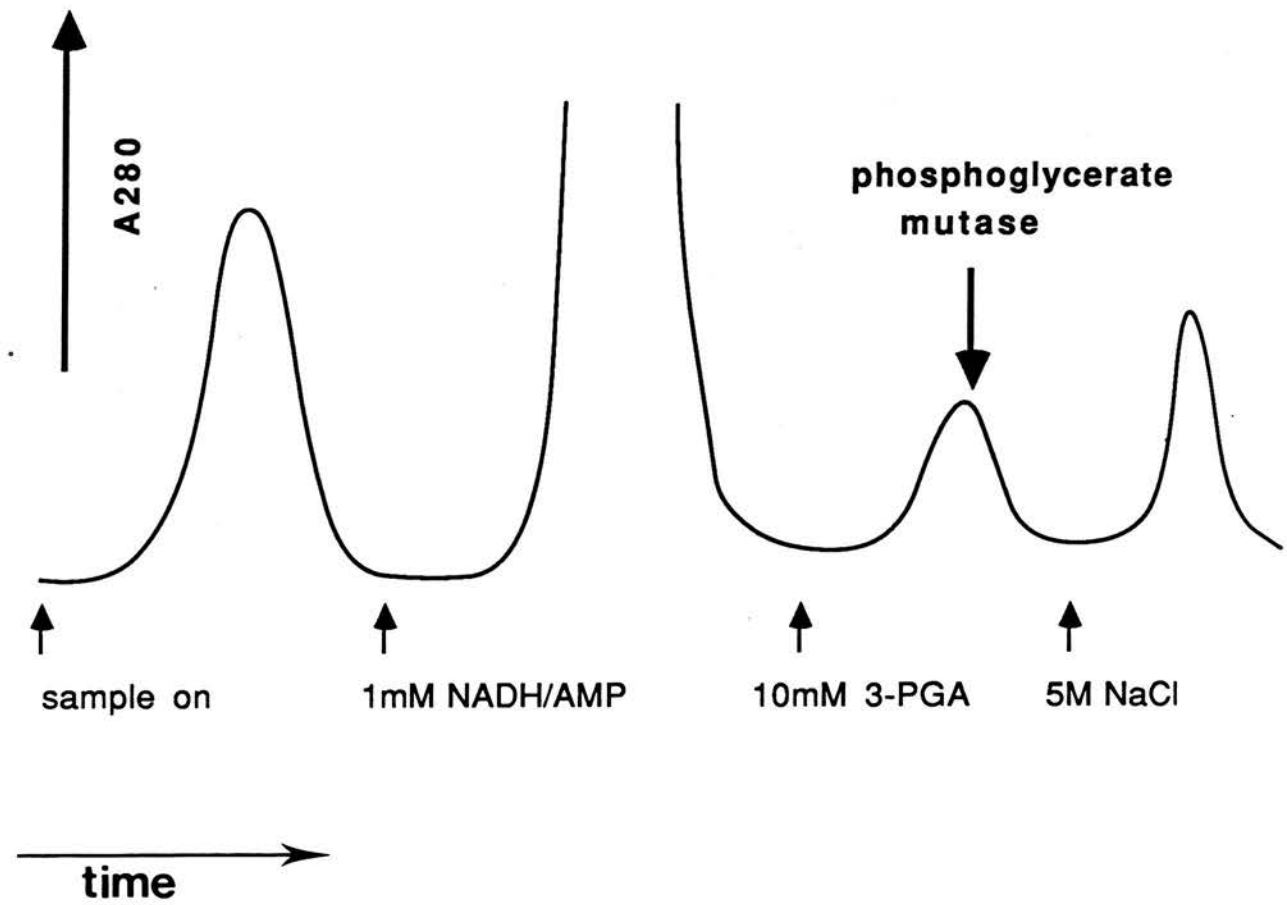

Figure 2.2. Schematic representation of a typical trace of absorbance at 280nm of the eluent from the cibacron blue-Sepharose column during the purification of phosphoglycerate mutase.

concentration and activity as described (2.2.12.3 and 2.2.12.4), then was made 80% ammonium sulphate saturated and stored at 4°C until required.

2.2.12.3 Determination of protein concentration

During purification of phosphoglycerate mutase, protein concentration was estimated by the method of Layne (1957). The absorbance of a suitably diluted sample of protein in a quartz cuvette was read at 280nm and 260nm against a water blank. The concentration of protein in mg/ml is given by the equation:

$$1.55x(A_{280}) - 0.76x(A_{260})$$

For pure samples of phosphoglycerate mutase, the extinction coefficient of the enzyme at 280nm was used to calculate the concentration of the enzyme. For yeast phosphoglycerate mutase, A_{280} 1.45 = 1mg.ml^{-1} protein.

2.2.12.4 Assay of phosphoglycerate mutase activity

During purification, the total phosphoglycerate mutase activity of samples was determined using the enolase-coupled assay described by Grisolia (1962).

Assay mix:

Tris-HCl pH7.0	50mM
$MgSO_4$	50mM

3-PGA	5mM
2,3-BPGA	0.1mM
enolase	0.08U
SDW	to 1ml

A sample of phosphoglycerate mutase was added to the assay mix in a quartz cuvette, mixed, and the rate of increase in absorbance at 240nm due to the formation of PEP was monitored in a recording spectrophotometer. One Enzyme Unit (1 EU) is defined as giving a rate of increase in absorbance at 240nm of 0.1/min. The phosphoglycerate mutase sample was first diluted so that <0.15 EU's were added to the assay. Pure yeast phosphoglycerate mutase has a specific activity of 7000-8000 EU's/mg (Price and Jaenicke, 1982).

2.2.12.5 Protease inhibitors:

The following protease inhibitors were added to cell slurries prior to breakage to prevent degradation of the enzyme :-

i) 3,4-Dichlorisocoumarin - inhibits serine proteases
stock solution - 5mM in DMSO, diluted x50 for use.

ii) 1,10-Phenathroline - inhibits metalloenzymes
stock solution - 100mM in DMSO, diluted x1000 for use.

iii) E64c - inhibits cysteine proteases
stock solution - 1mM in SDW, diluted x50 for use.

2.2.12.6 Denaturing gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli and Favre (1973). Protein samples were prepared by mixing with an equal quantity of boiling mix (10% (v/v) stacking gel buffer, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.005% (w/v) bromophenol blue), boiling for 3 min and centrifuging for 10s at high speed in a microfuge prior to loading.

2.2.13 Estimation of the kinetic parameters of wild-type and mutant phosphoglycerate mutases

2.2.13.1 Michaelis constants for 3-PGA and 2,3-BPGA

The Km's of wild-type and mutant phosphoglycerate mutases for the glycolytic substrate 3-phosphoglycerate and the cofactor 2,3-bisphosphoglycerate were estimated in an assay coupled through enolase, pyruvate kinase (PK) and lactate dehydrogenase (LDH) to the oxidation of NADH.

Assay mix:

Tris-HCl pH7.0	30mM
KCl	20mM
$MgSO_4$	5mM
ADP	0.2mM
NADH	0.15mM
enolase	0.08U

PK	0.5U
LDH	0.5U
3-PGA	varied
2,3-BPGA	varied
SDW	to 1ml.

The assay was started by the addition of a sample of phosphoglycerate mutase, diluted so as to give a maximum velocity not exceeding 2 nmoles NADH oxidised per minute. Under these conditions, the rate of reaction was determined solely by the activity of phosphoglycerate mutase. In experiments to determine the Km for 3-PGA, 2,3-BPGA was present at constant non-rate-limiting concentrations, and vice-versa. The reaction was monitored by the decrease in absorbance at 340nm, due to the oxidation of NADH, for 4 min in a Phillips PU 8720 UV/VIS scanning spectrophotometer at 30°C. Plastic UV cuvettes were used, and four cuvettes were monitored simultaneously. All measurements were carried out in triplicate, and standard deviations were calculated. Rates were converted to umoles NADH oxidised/min/umoles of subunits of phosphoglycerate mutase using the absorbance coefficient for NADH at 340nm of 6.2 $\mu M^{-1}.cm^{-1}$.

2.2.13.2 Estimation of the Michaelis constant for 2-PGA

The Km's of wild-type and mutant phosphoglycerate mutases for the gluconeogenic substrate 2-phosphoglycerate were determined using an assay coupled through phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to the oxidation of NADH.

Assay mix:

Tris, KCl, $MgSO_4$, NADH - as for 2.2.13.1.	
ATP	1mM
PGK	0.5U
GAPDH	0.5U
2,3-BPGA	0.1mM
2-PGA	varied
SDW	to 1ml.

The assay was carried out as described in section 2.2.13.1.

2.2.13.3 Synthase activity of phosphoglycerate mutase

A low 2,3-BPGA synthase activity has been identified in vertebrate phosphoglycerate mutases, and in one instance in yeast phosphoglycerate mutase (Sasaki et al., 1976). In order to detect any synthase activity in the wild-type and mutant forms of the yeast enzyme, the assay of Laforet et al., (1974) was used. This assay overcomes the problems caused by the inherent instability of 1,3-BPGA by continually synthesising this substrate from ATP and 3-PGA using the enzyme PGK. This reaction is driven by the continuous regeneration of ATP from ADP and creatine phosphate by the enzyme creatine phosphokinase (CPK). The addition of a sample of phosphoglycerate mutase leads to a complicated situation involving the interconversion of 1,3-BPGA, 2,3-BPGA, 3-PGA and 2-PGA. The synthesis of 2,3-BPGA should be favoured by the continual generation of the substrate 1,3-BPGA.

Reaction mix:

Tris-HCl pH7.0	0.2M
$MgCl_2$	2.5mM
ATP	1mM
creatine phosphate	30mM
3-PGA	4mM
CPK	25µg
PGK	1U
SDW	to 0.25ml.

The reaction mix was equilibrated in a microcentrifuge tube at 30°C for 5 min. A sample of phosphoglycerate mutase was added and mixed, and the assay was incubated at 30°C for 30 min, then boiled for 10 min (this hydrolyses any 1,3-BPGA, and largely inactivates the enzymes, although phosphoglycerate mutase is fairly heat resistant). 62µl cold 25% TCA was added and the tubes were vortexed and kept on ice for 30 min to ensure complete denaturation of the enzymes, then centrifuged at high speed for 10 min in a microcentrifuge. 150ul of the supernatant was removed and neutralised by the addition of 25µl 1.8M Tris-HCl pH8.0 and 8µl 3M NaOH to give a final pH of about 7.5. Control samples, in which the reaction was stopped at time zero by the addition of TCA, were also processed and served as blanks for the correction of any non-specific effects.

The amount of 2,3-BPGA produced in the above reaction was determined by monitoring its ability to activate phosphoglycerate mutase. Phosphoglycerate mutase activity was monitored using the enolase-coupled assay system (section 2.2.10.4) containing 5mM 3-PGA and no 2,3-BPGA. A standard curve was produced by adding known

concentrations of 2,3-BPGA to the assay system and monitoring the activity of 1 EU of phosphoglycerate mutase. This was found to be linear in the range 0-7 nmoles 2,3-BPGA, and is presented graphically in figure 2.3. The low activity observed in the absence of added 2,3-BPGA was probably due to a low level of contamination of Sigma grade I 3-PGA by 2,3-BPGA. Using this assay, 25µl aliquots from the neutralised, stopped synthase assay were used to determine the amount of 2,3-BPGA produced by each sample of enzyme studied.

2.2.13.4 Phosphatase assay

The 2,3-BPGA phosphatase activity of phosphoglycerate mutase samples in the presence and absence of the activator 2-phosphoglycollate was measured using the following assay system:

a) Phosphatase Reaction mix:

Tris-HCl pH7.0	50mM
2-P-glycollate	0 or 1mM
2,3-BPGA	0.2mM
SDW	to 1ml.

Procedure:
The reaction mix was equilibriated in a microcentrifuge tube for 5 min at 30°C, then a sample of the phosphoglycerate mutase to be studied (2-20µg) was added and the reaction was allowed to proceed for 1 hr at 30°C. 200µl cold 25% TCA was added to stop the reaction and the tubes were kept on ice for 10 min, then centrifuged at high speed for 10 min at 4°C. Control samples, in

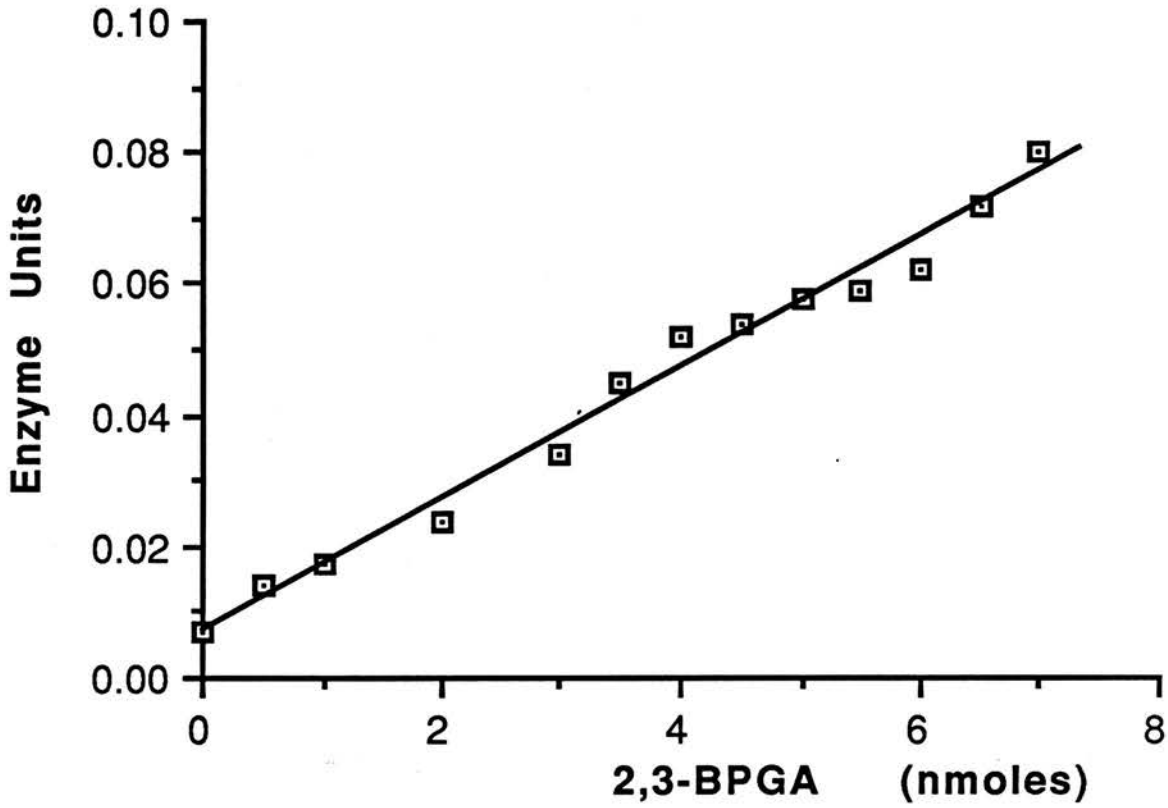

Figure 2.3. Standard curve obtained for the determination of the amount of 2,3-bisphosphoglycerate formed in the synthase assay.

which the reaction was stopped at time zero by the addition of TCA, were used as blanks in order to correct for any interfering effects.

b) Determination of Pi:

The amount of inorganic phosphate released in the phosphatase reaction was measured using the assay of LeBel et al., (1978).

Solutions:

A: 0.25% copper sulphate pentahydrate
4.6% sodium acetate trihydrate
in 2N acetic acid.

B: 5% ammonium molybdate

C: 2% Elon (p-methyl-aminophenol sulphate)
5% sodium sulphite

Procedure:

0.5ml of the TCA supernatant of the sample was placed in a 5ml Falcon tube. 1.5ml of solution A and 250µl of solution B were added and mixed thoroughly, then 250µl of solution C was added followed by further mixing. The tubes were allowed to stand for at least 7 min to allow colour to develop, and absorbance was then read at 870nm. A standard curve for Pi was constructed using acidified KH_2PO_4 of known concentrations in triplicate. Mean values together with SD's are plotted in figure 2.4. The standard curve was found to be linear in the range 0-7µg Pi.

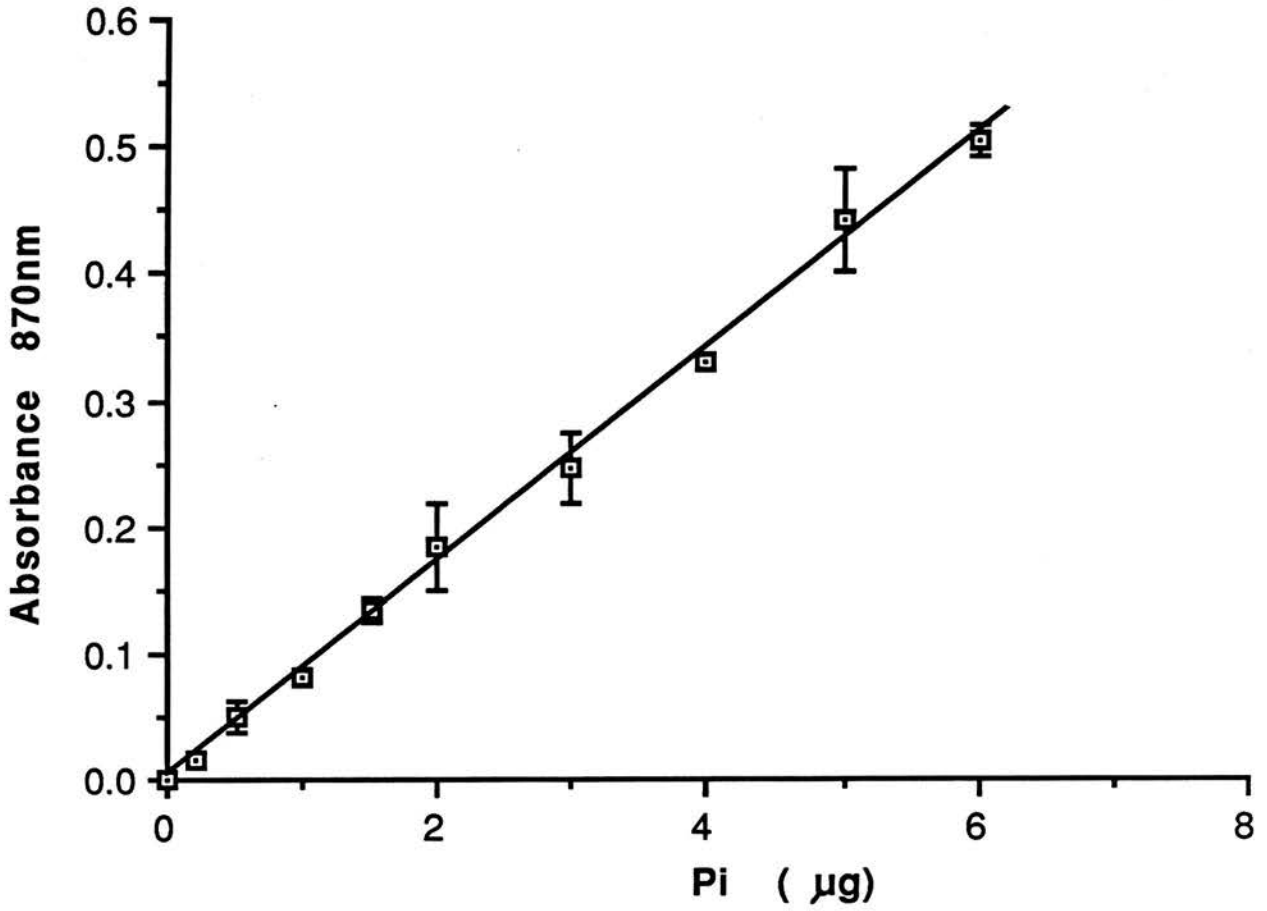

Figure 2.4. Standard curve obtained for the quantitation of inorganic phosphate released in the phosphatase assay.

2.2.14 Computer programs

a) Sequence analysis and manipulation:

The University of Wisconsin Genetics Computer Group (UWGCG) suite of programs were used for the analysis and manipulation of DNA and protein sequences (Devereux et al., 1984).

b) Protein structure modelling and display:

FRODO, written by Alwyn Jones and converted to run on a VAX under VMS on the Evans and Sutherland PS300, was used for the display and modelling of protein structures.

c) Statistical analysis and presentation of data:

The program DNRP53 (Duggleby, 1984) was used for non-linear regression analysis of data. CRICKETGRAPH, run on an Apple Macintosh microcomputer, was used for the graphical presentation of data.

3. Localisation and Sequencing of the GPM gene.

This chapter describes the localisation of the GPM gene on the plasmid YEP13.GPM supplied by Professor Dan Fraenkel and the subsequent sequencing of the gene.

3.1 Restriction mapping and location of the yeast GPM gene locus

The plasmid shown by Fraenkel (Kawasaki and Fraenkel, 1982) as capable of complementing a gpm- strain of yeast consisted of the shuttle vector YEP13 with a 6kb insert of yeast chromosomal DNA in the unique BamHI site. Before sequencing, the GPM gene locus had to be located and mapped by restriction analysis. A large-scale preparation of the plasmid was carried out and 500ng aliquots were digested using the restriction enzymes BamHI, BglII, EcoRI, HindIII, SalI and XhoI singly and in pairs. Once digestion was complete, the restriction fragments were separated on a large agarose gel (1%, containing EtBr). Several digests of lambda DNA were also run to act as markers. The gel was inspected on a UV transilluminator, and when good separation of the fragments was observed the DNA was transferred on to a Hybond-N filter by Southern blotting. This filter was first hybridised with a mixture of YEP13.GPM and lambda DNA labelled with α-^{32}P-dATP by random priming, washed in stringent conditions (0.1xSSC, 65°C) and autoradiographed overnight at -70°C. These probes hybridised with every DNA fragment on the filter (fig. 3.1a), and by comparison with the R_F values of the lambda DNA fragments each YEP13.GPM fragment was assigned an approximate size in base-pairs. Using the

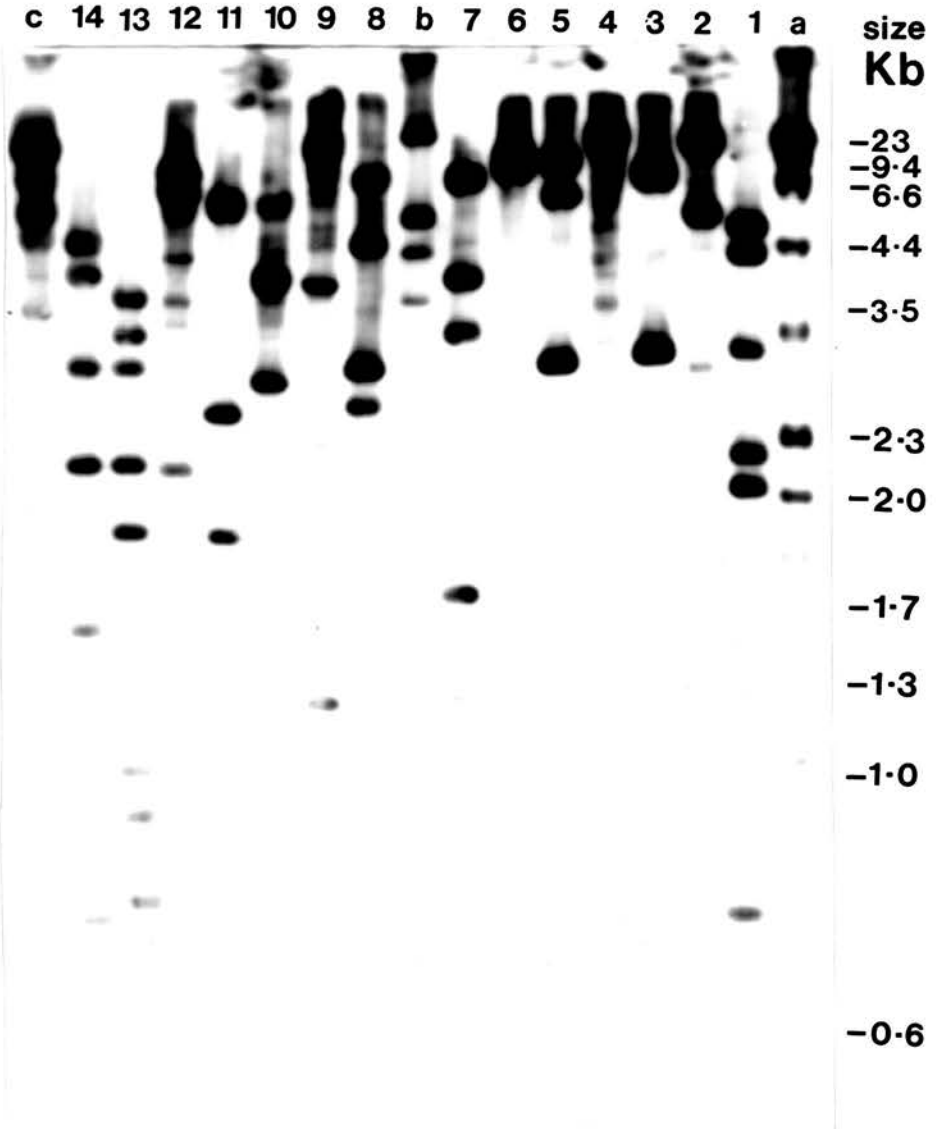

Figure 3.1a. Restriction mapping of YEP13.GPM. Autoradiograph of a Southern blot of YEP13.GPM cut with the following restriction enzymes:

1. EcoRI	4. BamHI	7. SalI + HindIII	11. XhoI + BglII
2. HindIII	5. BglII	8. BamHI + SalI	12. XhoI + BamHI
3. SalI	6. XhoI	9. BamHI + HindIII	13. EcoRI + SalI
		10. BamHI + BglII	14. EcoRI + HindIII.

Lanes a,b,c contain lambda DNA cut with HindIII, HindIII + EcoRI, and EcoRI, respectively. YEP13.GPM and lambda DNA were used as probes.

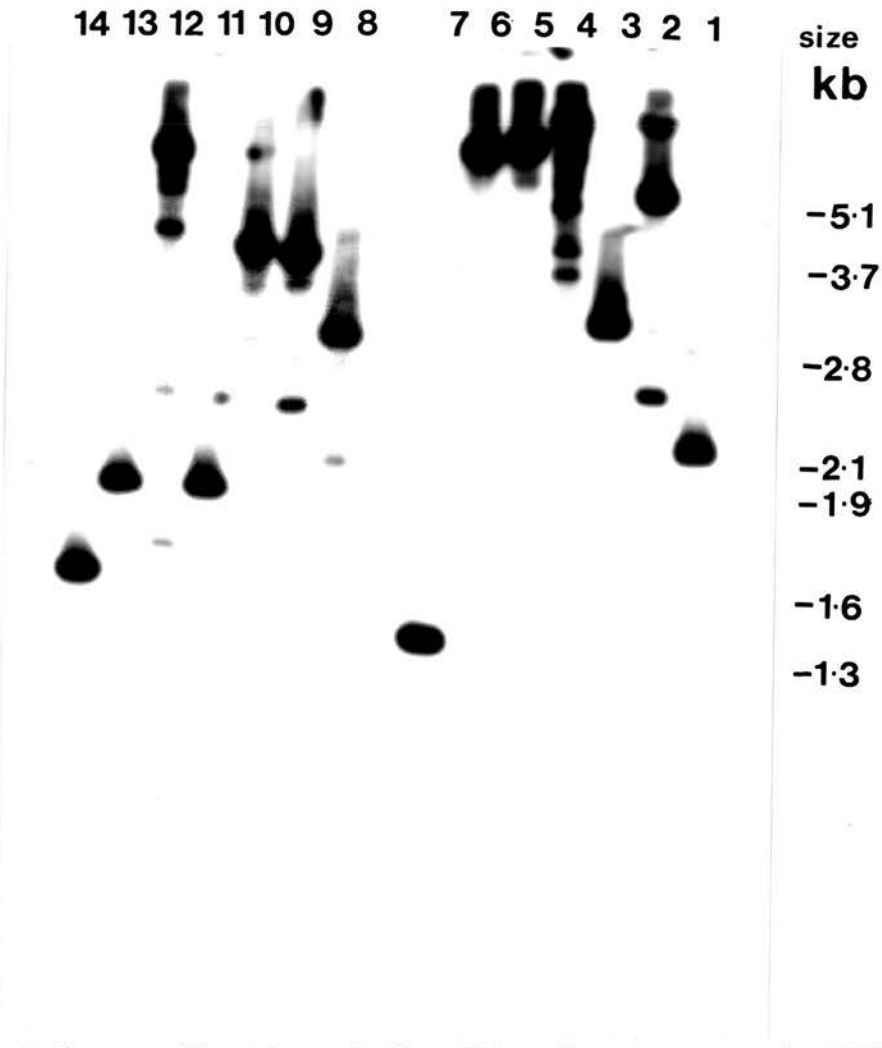

Figure 3.1b. Localisation of the GPM coding sequence in YEP13.GPM. The Southern blot from figure 3.1a was probed with oligonucleotides derived from the amino acid sequence of the yeast phosphoglycerate mutase enzyme. The oligonucleotides hybridised to one major fragment in each lane. Some non-specific background hybridization is also observed.

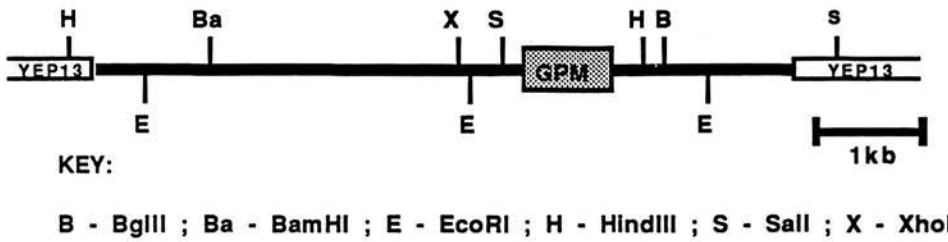

Figure 3.1c. Restriction map of the yeast chromosomal insert of YEP13.GPM. The location of the GPM gene, as determined by Southern blotting experiments, is indicated.

information obtained from the single and double digests, in association with the known restriction map of YEP13, a map of the yeast DNA insert was constructed (fig. 3.1c).

From our knowledge of the protein sequence it was apparent that the GPM gene would be less than 1kb in length (assuming no introns, which are very rare in S.cerevisiae). Accordingly, the next step was to narrow down the locus of the GPM gene in the insert. The filter was stripped of hybridized probe and reprobed using an oligonucleotide designed from the N-terminus of the protein (this was originally synthesised in an attempt to isolate the gene). The oligonucleotide was first end-labelled with γ-^{32}P-ATP then hybridised with the Hybond filter in 6xSSC for 30 min. The filter was then washed in 6xSSC at 45°C and autoradiographed overnight at -70°C. The oligonucleotide hybridised most strongly to one band in each of the YEP13.GPM digests (figure 3.1b). Referral to the restriction map showed that all these bands contained a 1.3kb fragment bounded by a HindIII and a SalI site. This experiment was repeated using an oligonucleotide designed from the C-terminus of the protein, with the same result. These observations indicated strongly that the GPM gene resided on a 1.3kbp HindIII/SalI fragment in the insert (figure 3.1c).

3.2 Subcloning and sequencing of the GPM gene

The location of the GPM gene on a 1.3kbp HindIII-SalI fragment allowed DNA sequencing to be used. The 1.3kbp fragment was thus subcloned into the phage M13mp19 to enable sequencing of the GPM

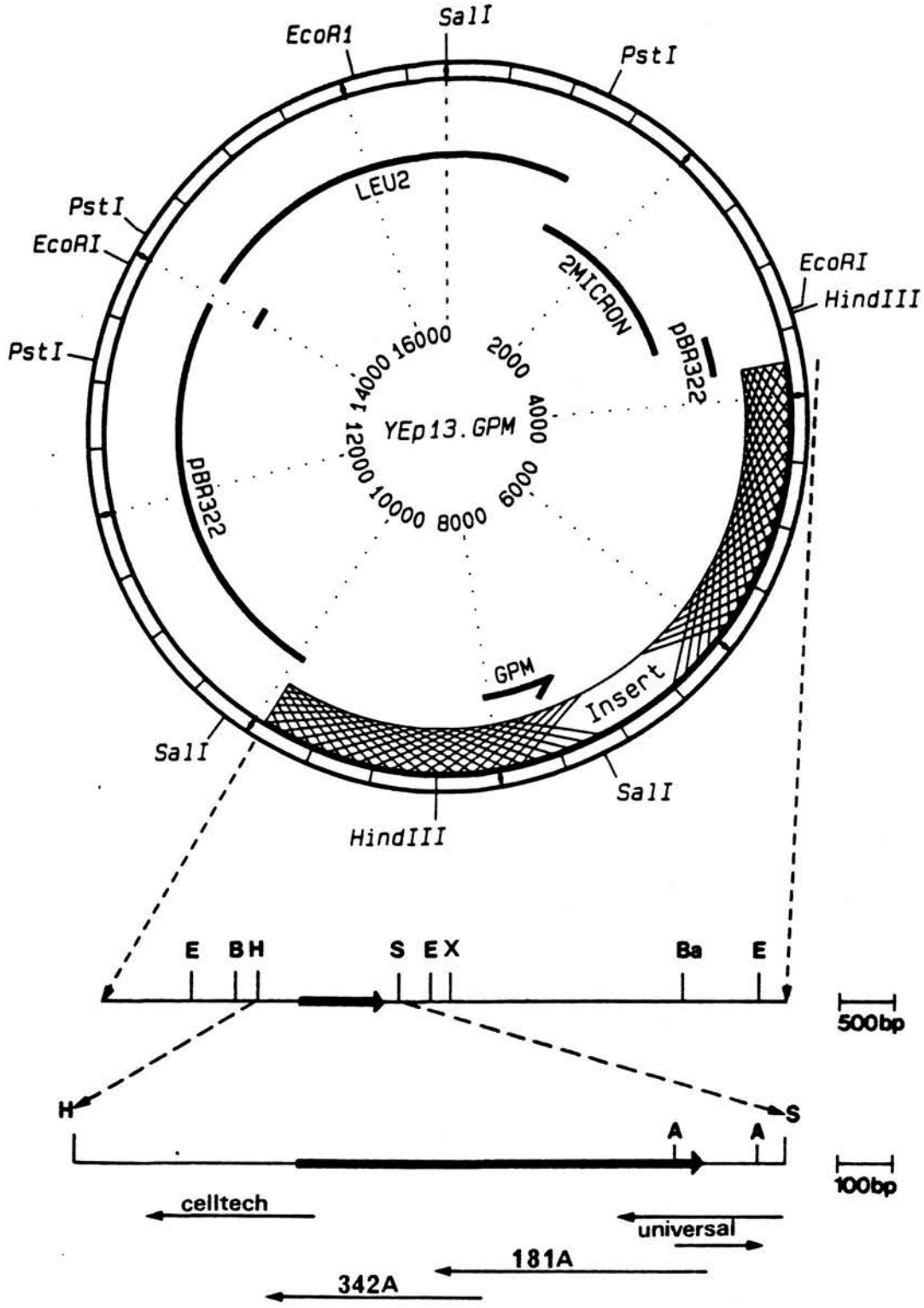

Figure 3.2. Restriction map of YEP13.GPM and sequencing strategy of the GPM gene. The thick lines indicate the GPM coding sequence. Arrows show the direction and length of individual sequence determinations. Oligonucleotides used as sequencing primers are indicated. Restriction enzymes: H - HindIII; S - SalI; E - EcoRI; B - BglII; Ba - BamHI; X - XhoI; A - AluI.

gene. Subcloning into M13mp18, which contains the polylinker in the opposite orientation, was also attempted; but in this orientation the insert appeared deleterious to the phage as yields of ssDNA were always very poor. The subcloning and sequencing strategy is summarised in figure 3.2. The Sequenase™ DNA sequencing kit was used to allow extended readthrough from each primer. Initially the universal primer, which hybridises near the SalI site of M13mp19, was used as a sequencing primer. This allowed the location of the 3' end of the GPM coding sequence, which terminated about 150bp upstream of the SalI site, to be identified. The translated amino acid sequence of this 'tail' region differed significantly from the previously reported protein sequence (Fothergill and Harkins, 1982), (discussed later). The fidelity of the DNA sequence was confirmed by the isolation of an AluI restriction fragment in M13mp9 which allowed sequencing of the complementary DNA strand for this region. This sequence matched that from the other strand, vindicating the DNA-derived sequence. In order to extend the sequencing of the GPM gene, the oligonucleotide 181A was designed to act as a sequencing primer. This oligonucleotide hybridised 150 bases in from the SalI site in MW1 and allowed another 350 bases of coding sequence to be read. A further sequencing primer, oligonucleotide 342A, enabled the final 350 bases of coding sequence to be read. Finally, an oligonucleotide synthesised by Celltech ('celltech' oligonucleotide), and designed as a probe for the isolation of the GPM gene from a lambda library was found to hybridise to MW1 at around codon 13 of the GPM coding sequence. This primer allowed about 250 bases of the 5' flanking region to be sequenced. A total

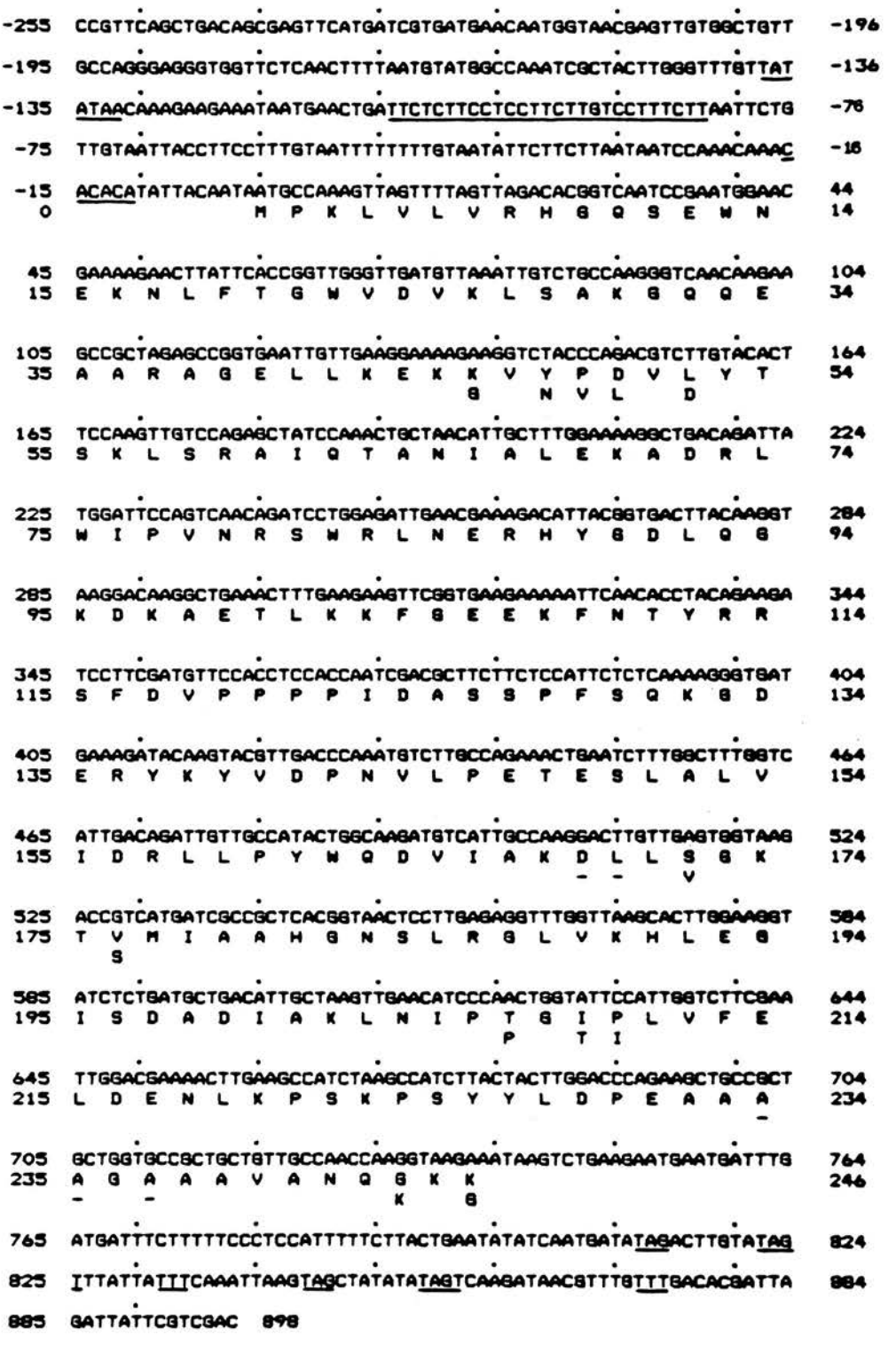

Figure 3.3. Nucleotide sequence and derived amino acid sequence of of 1154bp were sequenced, comprising a 741bp open reading frame encoding the enzyme phosphoglycerate mutase plusthe GPM gene. The underlined sequences are discussed in the text. Residues different in the published protein sequence are indicated below the deduced amino acid sequence. The DNA sequence has been lodged with the EMBL sequence database, accesion number X06408.

of 1154bp were sequenced, comprising a 741bp open reading frame encoding the enzyme phosphoglycerate mutase plus some 5' and 3' flanking sequences (figure 3.3).

3.3 Features of the DNA sequence

Phosphoglycerate mutase is typical of glycolytic enzymes in yeast in being highly expressed. This has a number of consequences for the DNA sequence. Most striking is the extreme codon bias exhibited by the GPM gene. Only 31 codons are used, and of these 24 account for 93% of the amino acids. This compares with values of 94% for phosphoglycerate kinase, 95% for enolase I and 95% for alcohol dehydrogenase I (Bennetzen and Hall, 1982). Figure 3.4 shows the three reading frames of the GPM gene scanned for codon bias using the UWGCG program CODONPREFERENCE. This program compares the test sequence against a table of the codons used in seven highly expressed yeast genes coding for other glycolytic enzymes. The results are striking, with a high degree of bias coinciding with a 741bp open reading frame (boxed) in the second reading frame. Rare codons (used <10% of the time) are indicated by a dash. Other features of the GPM sequence are also typical of highly expressed genes in yeast. These include A residues at positions -1 and -3 and a 44 base A-rich region containing no G residues immediately adjacent to the initiating ATG codon (Hamilton et al., 1987). A CT-rich block, here present at -83 to -109, is also common in highly expressed genes, although the CAAG sequence normally associated with such blocks is absent (Dobson et al., 1982).

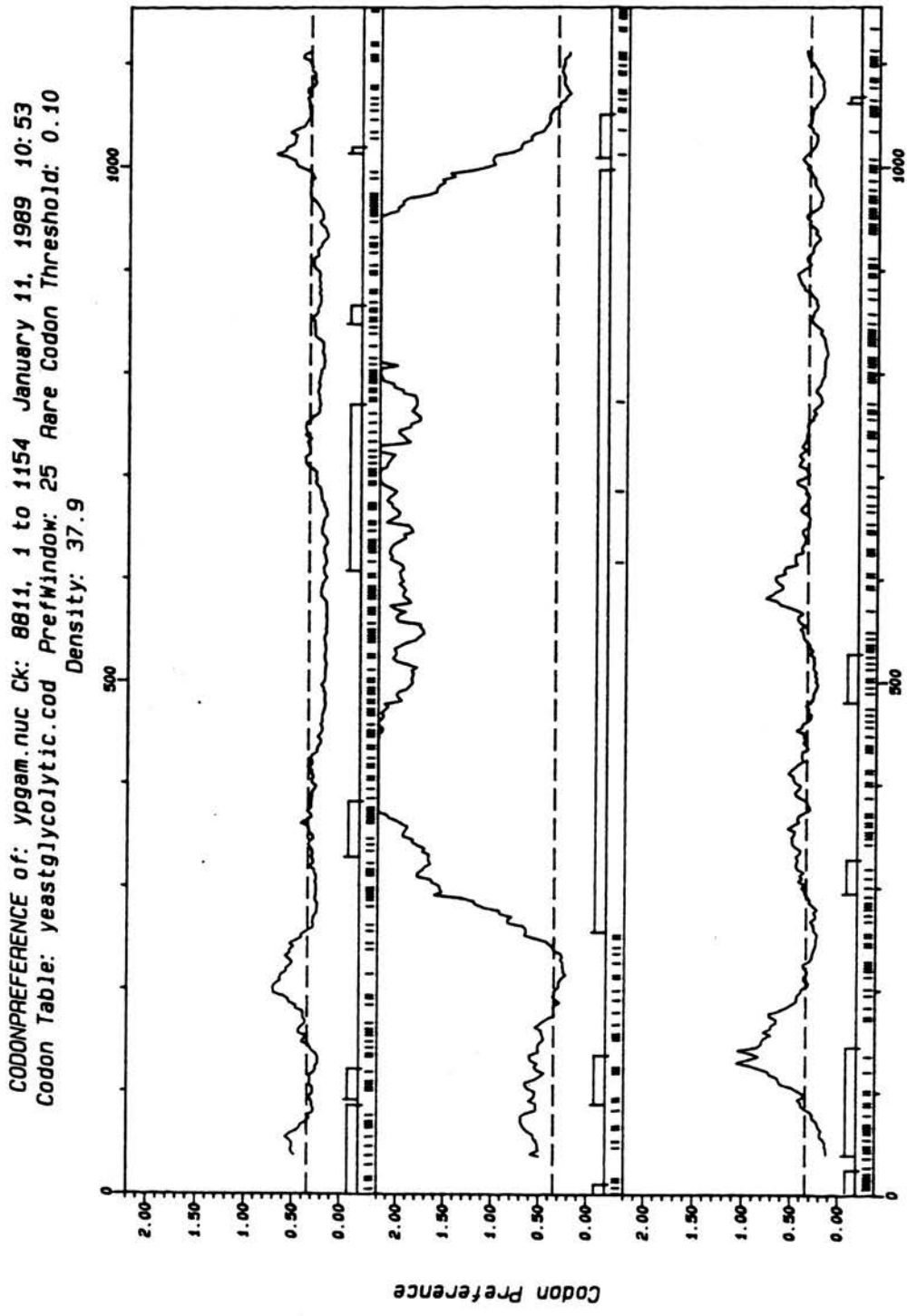

Figure 3.4. Codon bias exhibited by the GPM gene. The three reading frames of the GPM gene were scanned for degree of codon bias, using seven highly expressed yeast glycolytic genes for comparison. Boxes indicate open reading frames, and the ORF of the GPM gene is present in the centre panel. Codons used less than 10% of the time are marked with dashes.

Transcription may initiate at the TATA box at position -138, and the sequence CACACA at position -16 has been found in several other yeast genes, although its function is unclear as yet (Dobson et al., 1982). The 3' flanking sequence is 73% AT rich, and contains two possible transcription termination signals TAG...TAGT...TTT starting at positions 812 and 844 (Zaret and Sherman, 1982).

3.4 Features of the protein sequence

The protein sequence deduced from the DNA sequence largely confirms that previously determined by Edman degradation of proteolytic fragments. There are several discrepancies, as indicated in figure 3.4, and the reasons for these have been discussed by White and Fothergill-Gilmore (1988). Briefly, they reflect both strain differences and problems inherent in manual protein sequencing. However, none of these discrepencies significantly alter our ideas about the structure/function relationship of the enzyme - although the C-terminal tail appears to be longer and more flexible than was previously envisaged.

In summary, the gene encoding the enzyme phosphoglycerate mutase from yeast has been identified, mapped and sequenced using standard techniques. The gene exhibits a high degree of codon bias, and several possible transcription/translation control elements have been identified. The DNA-derived protein sequence largely confirms the previously published amino acid sequence.

The work described in this chapter has been published (White and Fothergill-Gilmore, 1988). A reprint of the manuscript is included at the rear of this thesis.

4.Replacement of the GPM gene

4.1 Why gene replacement?

The purpose of this project was to design, express and characterise mutant forms of the enzyme phosphoglycerate mutase. These mutant enzymes were to be expressed from plasmids in yeast, which normally possesses a single wild-type chromosomal copy of the GPM gene, and therefore expresses wild-type phosphoglycerate mutase. Obviously it was undesirable to have two forms of the enzyme being expressed in the same cell because the altered properties of the mutant form could be blurred or masked by the presence of the cell's own wild-type mutase. Accordingly, it was decided to create a yeast strain devoid of any wild-type mutase as a vehicle for the expression of engineered mutases.

Two possibilities existed; to disrupt the GPM gene or to remove it entirely. S.cerevisiae has an efficient DNA double-strand-break repair system which can be utilised to promote homologous recombination between the yeast genome and incoming linear DNA fragments. DNA constructs can be designed in vitro which will result in the disruption or replacement of target genomic sequences when introduced into yeast cells. Generally a marker gene such as LEU2 or HIS3 is integrated into the genome, allowing positive selection for the integration event by plating out cells on the appropriate omission medium. The marker gene can either disrupt or replace the target genomic sequence. The two options are of equal experimental complexity (indeed the methodologies are identical)

and would each give rise to a strain of yeast with identical phenotype. There was however one important consideration which made gene replacement the better option in this case.

When a gene is disrupted, it ceases to express a functional protein, but all the genetic information is still present in the cell. If a mutant GPM gene on a plasmid were introduced into a GPM-disrupted strain of yeast, then there would be a remote chance that homologous recombination would occur, reconstituting the wild-type GPM gene on the plasmid. Although rare, this event might confer a selective advantage to the cells, such as increased ability to utilise glucose, which would allow them to outgrow their mutant siblings. In this way any retro-conversion could quickly establish itself in a population of growing yeast. Undetected, this would jeopardise the characterisation of mutants; periodic checking would be tedious. Gene replacement, on the other hand, removes all the coding sequence, eliminating the possibility of reconstitution of the wild-type gene.

4.2 Strategy for gene replacement

The yeast strain DBY747 (GPM+ his3-) was chosen as the parental strain for gene replacement. In order to replace the GPM gene, it was necessary to construct a plasmid which carried the HIS3 gene flanked by DNA sequences which flank the GPM gene on the yeast chromosome. When this plasmid was linearised and introduced into yeast, the flanking sequences on the plasmid and the chromosome could undergo homologous recombination (figure 4.1). A number of

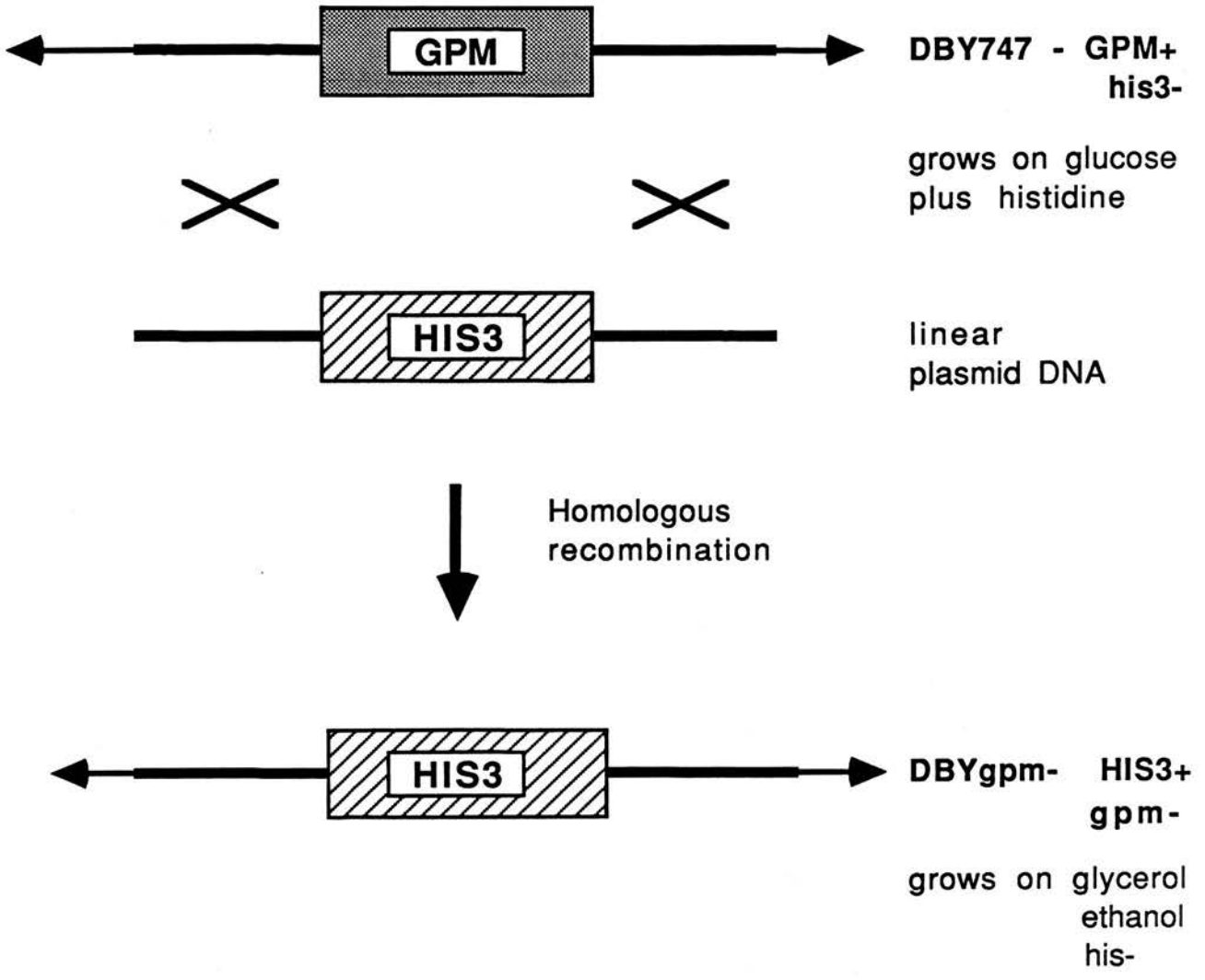

Figure 4.1. Schematic outline of the process of replacement of the GPM gene with the HIS3 gene in the yeast strain DBY747.

cells would gain a chromosomal copy of the HIS3 gene, with the concommitant loss of their GPM gene. These cells could be selected for by plating the transformants onto his- selective plates.

The plasmid construction is summarised in figure 4.2, and consisted of two main steps. Firstly, the BamHI / SalI fragment of YEP13.GPM was cloned into pUC18, followed by the SalI fragment which carries the GPM gene. This resulted in the reconstruction of the GPM locus in plasmid pUC-GPM. Secondly, the GPM gene was removed from pUC-GPM by digestion with XhoI / BglII, and the HIS3 gene (from the plasmid YIP1) on a XhoI / BamHI fragment was ligated in its place, creating the plasmid pUC-HIS3. This construct was then linearised by cutting with the enzymes BamHI and SalI and the restriction mix containing 3µg DNA was transformed into the yeast strain DBY747 by LiAc transformation. The transformation mix was plated out on YOGE his- plates to select for transformants (gpm- cells lose the ability to grow on glucose, and must be grown with glycerol and ethanol as carbon sources). After 7 days at 30°C 25 transformants were obtained. The phenotype of these transformants was gpm- HIS3+ as expected. They grew happily on YGE plates but not at all on YD plates. The expected genomic maps of the parental and derived strains are shown in figure 4.3.

To ascertain whether or not gene replacement had occurred, a Southern blot was prepared in the following manner. Genomic DNA was prepared from DBY747 and from one of the transformants, provisionally designated DBYgpm-. 10µg of DNA from each strain was digested with the enzymes HindIII and SalI at 37°C overnight.

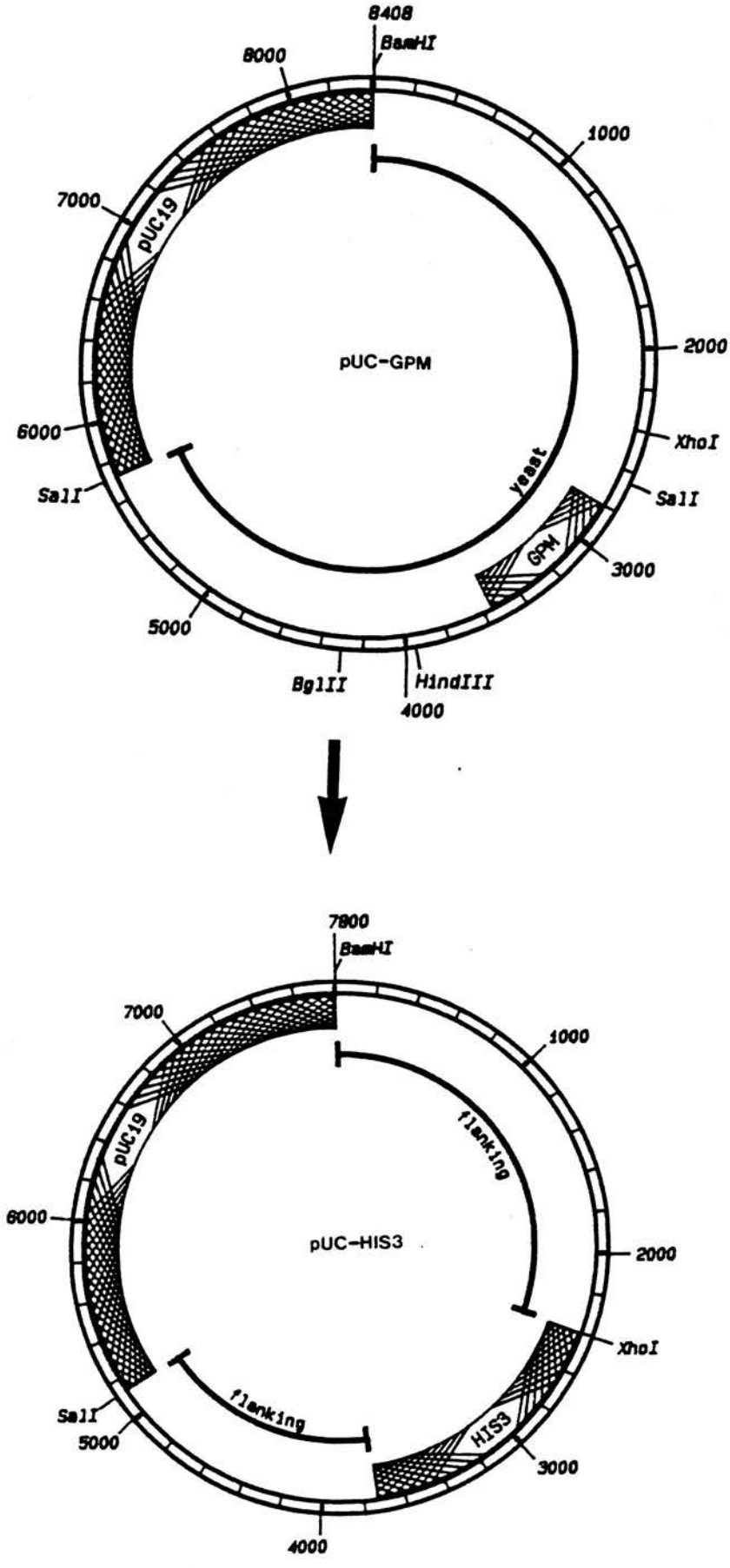

Figure 4.2. Plasmid construction required for gene replacement.

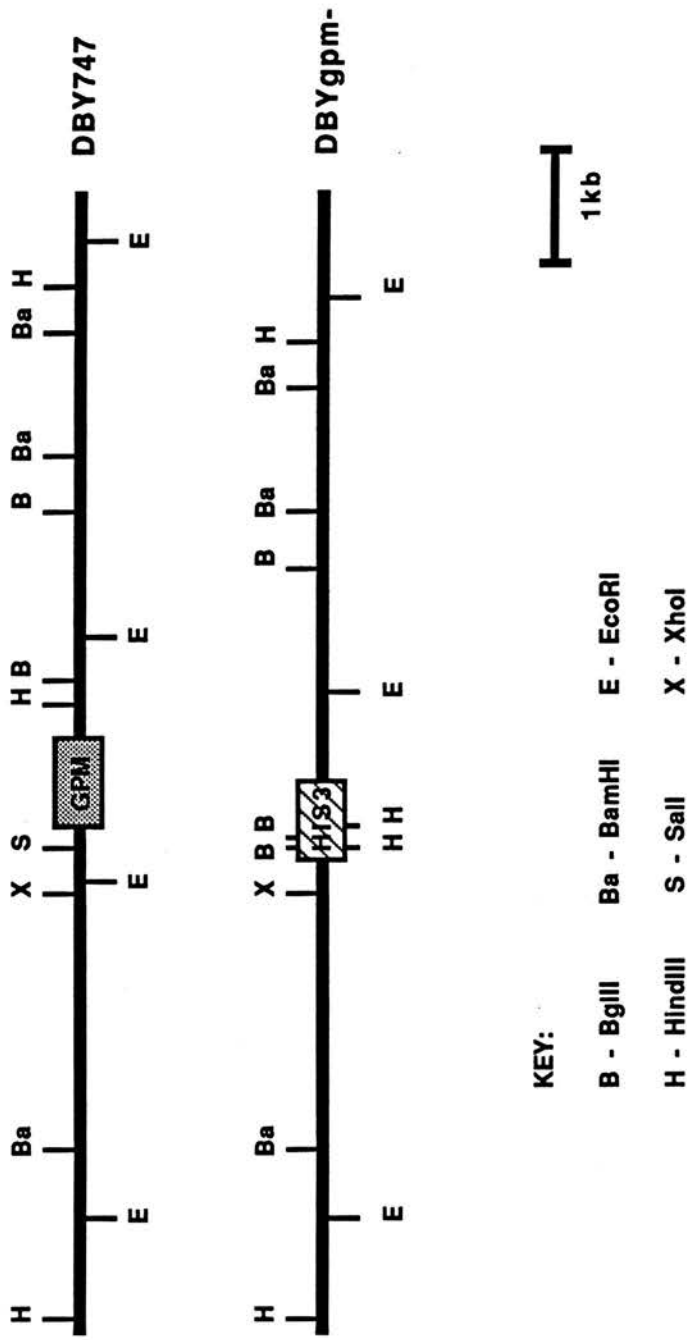

Figure 4.3. Genomic map of DBY747 and predicted genomic map of the strain DBYgpm- derived from gene replacement. The positions of the GPM and HIS3 genes are indicated.

These digests were then separated by electrophoresis on a large 1% agarose gel, along with DNA fragments containing the GPM and HIS3 genes as markers. The DNA was transferred onto Hybond-N by Southern blotting, and was hybridised first with the 1.3kbp HindIII/SalI fragment of YEP13.GPM, which contains the GPM gene, labelled by random priming with α-^{32}P-dATP. After hybridisation, the filter was washed under stringent conditions (0.1xSSC, 65°C, 15 min) and autoradiographed overnight. It was then stripped of all hybridised probe and the process above was repeated using a DNA fragment from YIP1 containing the HIS3 gene as probe. The results of this experiment are shown in figure 4.4. As expected the GPM probe hybridised with a 1.3kb band in the DBY747 track, but no band in the DBYgpm- track. Conversely the HIS3 probe detected no like sequences in the DBY747 track, but hybridised with three bands in the DBYgpm- track. The genomic map predicted for DBYgpm- indicates that a HindIII/SalI digest should generate two fragments of 4.3 and 4.2kbp containing HIS3 sequences. This doublet is present on the autoradiograph, although the bands have merged. The two spurious bands present are not predicted by the genomic map, but may be due to incomplete digestion of the DNA, 'star activity' of one of the enzymes or contaminating sequences in the HIS3 probe. Nevertheless it is clear that the gene replacement has been successful.

In summary, the creation of a gpm- strain of yeast was a prerequisite for the expression and analysis of mutant forms of phosphoglycerate mutase. This was acheived by gene replacement, and the derived strain DBYgpm- was shown to conform to the expected genotype and phenotype.

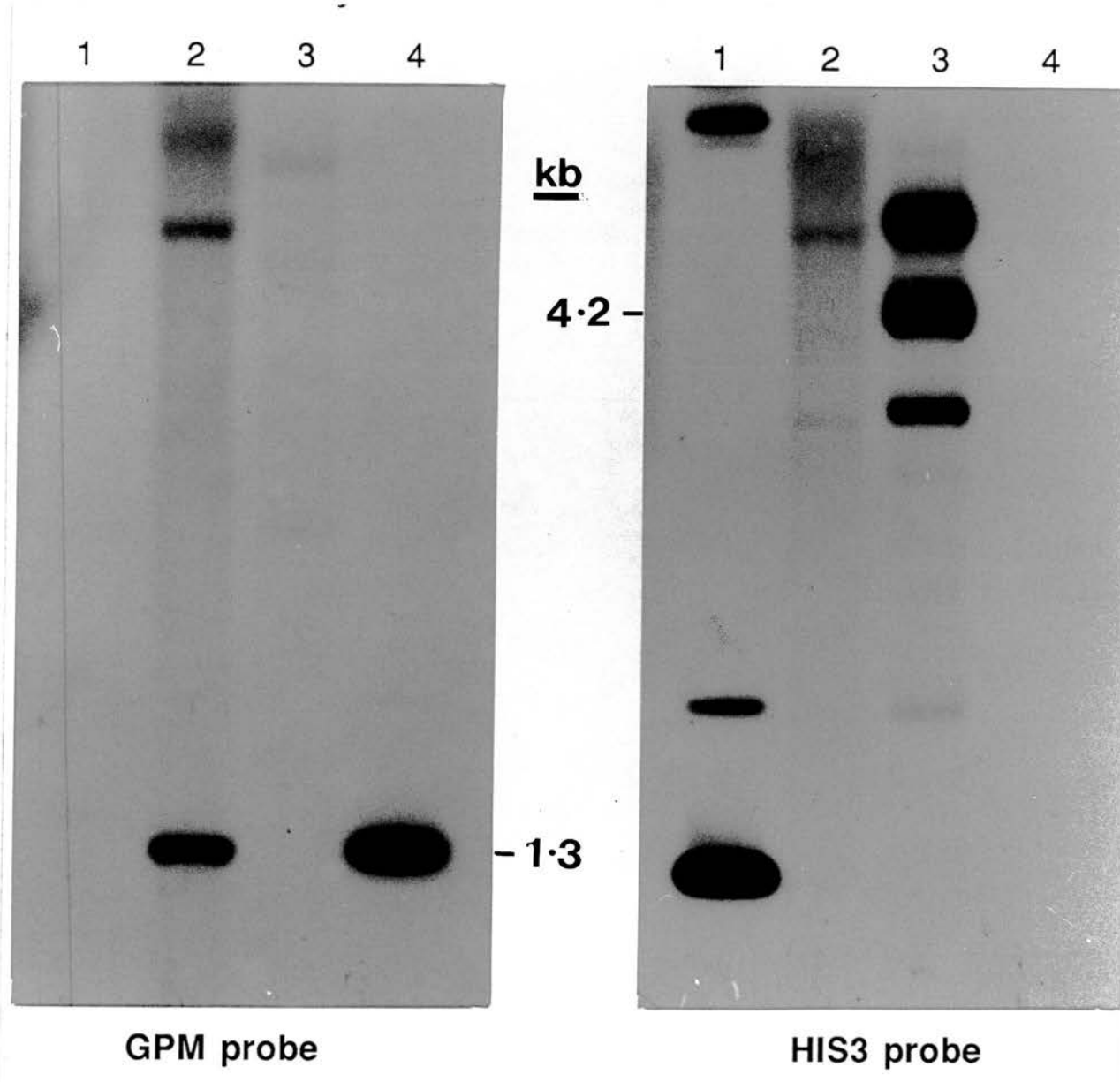

Figure 4.4. Southern blot analysis of gene replacement.

1. HIS3 marker
2. DBY747 genomic DNA cut with HindIII and SalI
3. DBYgpm- genomic DNA cut with HindIII and SalI
4. GPM marker.

Experimental details are described in the text.

5. Design, Construction and Expression of mutant GPM's.

Once a GPM deleted strain of yeast had been created, the next step was to produce mutant forms of phosphoglycerate mutase for analysis. Two mutations likely to prove informative were chosen. One was the replacement of serine 11 with a glycine (S11G), which would probe the molecular basis of the observed catalytic differences between mutases and synthases. As discussed in the introduction (section 1.10), residue 11 is a serine in all mutases so far sequenced and is likely to be involved in the binding of the substrates and cofactor. In synthases however, this residue is always a glycine, and could perform no role in bonding. Would the S11G mutation make yeast phosphoglycerate mutase less of a mutase and more of a synthase? Secondly, it was decided to investigate the role of the C-terminal 'tail' by changing lysine 246 to a glycine (K246G). This would abolish one of the positive charges on the tail which have been suggested as important for substrate and cofactor binding (section 1.11). What effect would the loss of this charged group have on the three activities of the enzyme? This section describes the design, creation and expression of the two mutants.

5.1 Design and construction of the mutants

In order to effect the mutations discussed, the Eckstein method of site-directed mutagenesis was employed. This method, supplied in kit form by Amersham, is highly efficient and is carried out in vitro, thus abolishing the risk of incorporating spurious mutations during DNA replication in repair-deficient cell lines. Firstly, two oligonucleotides were designed to act as mutagenic primers.

Each was an 18-mer and contained the 3-base substitution necessary to produce the required mutation whilst preserving the strong codon bias. The two oligonucleotidess are shown below, with the differences in the wild-type sequence highlighted:

W-T **GGA**

S11G 5'- TT CCA TTC ACC TTG ACC G - 3'

W-T **TTT**

K246G 5'- CA GAC TTA ACC CTT ACC T - 3'

The two mutants were produced in parallel; in each case the M13mp19 clone MW1, which contained the entire GPM gene on a 1.3kbp HindIII/SalI insert, was used as a template for mutagenesis. The double-stranded phage DNA obtained from the in vitro mutagenesis was transformed into competent TG1 cells; transformants were plated out in top agar on a lawn of TG1 cells and the plates were incubated at 37°C overnight to allow plaques to form.

5.2 Screening for the mutants

The plaques obtained from the mutagenesis experiments could be one of two things - mutant or wild-type. If the mutagenesis had worked successfully, then up to 90% of clones should be mutant (according to Amersham anyway!). The following screening protocols were used to check for the two mutations:

a) S11G:

100 plaques were obtained from the transformation with the DNA from the mutagenesis reaction. Ten of these plaques were picked and small single-stranded phage preparations were obtained from each one. Preliminary screening was carried out by C-tracking the ten clones (i.e. only the C-track of the sequencing reaction was run) to check for the presence of the two extra C-bands introduced by the mutation. The celltech oligonucleotide, which hybridised very close to the area mutagenised, was used as a sequencing primer, and the sequencing gel was run for only one hour. Although inconclusive due to the close proximity of the primer to the area to be sequenced, the C-tracking suggested that at least three of the clones contained the expected extra bands. These three clones were sequenced in all four tracks, this time using the oligonucleotide 342A as a more distant primer and running the gel for 4.5 hours. All three clones clearly carried the expected GGA->ACC substitution.

b) K246G:

About 200 plaques were obtained and ten were grown up to provide ssDNA for sequencing. As this mutation would result in the loss of three T residues, T-tracking was chosen for preliminary screening of the clones. The oligonucleotide 181A, which hybridises just outside the region of mutagenesis, was used as a primer, and the gel was run for about one hour. Again, due to the close proximity

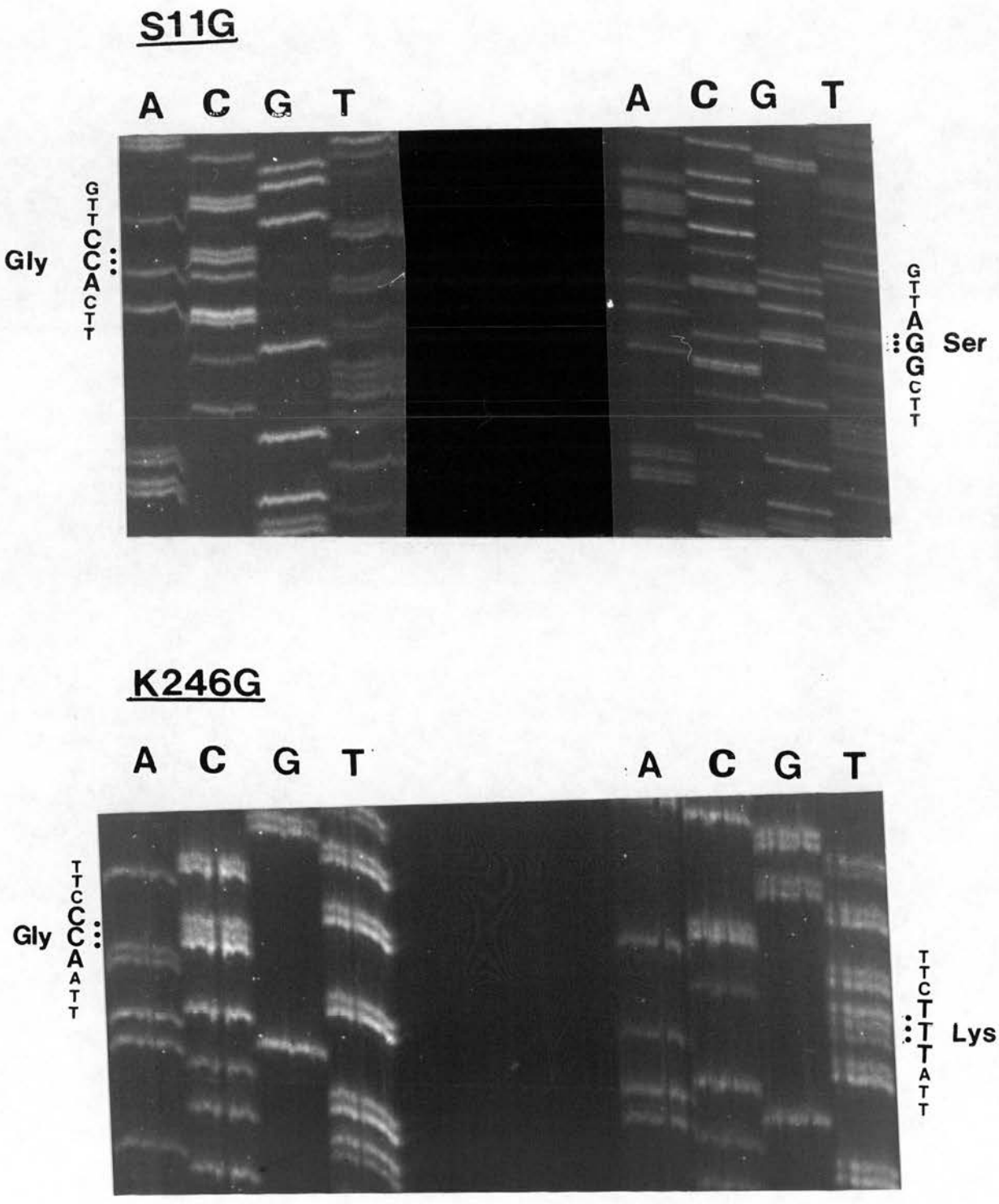

Figure 5.1. DNA sequence analysis of the S11G and K246G mutants in phage M13. In each case the corresponding wild-type sequence is shown on the right of the mutant sequence.

of the primer, the autoradiograph obtained was not unequivocal, but it appeared that all ten clones lacked the three T residues. Three of the clones were sequenced in all tracks, using the universal primer and running the gel for 3 hours. All three clones carried the correct mutation TTT->ACC. Autoradiographs of both mutant sequences, alongside the corresponding wild-type sequences, are shown in figure 5.1.

These results suggested that mutagenesis had been accomplished with fairly high efficiency. Direct sequencing of a few clones would probably yield the required mutant, eliminating the need for tedious screening procedures.

5.3 Subcloning of the mutants

Both the desired mutant GPM genes had now been created in bacteriophage M13. However, in order to express the mutant enzymes in yeast it was necessary to subclone the mutated GPM genes back into a yeast/E.coli shuttle vector. The vector pJDB207 was chosen as it allowed one simple cloning step, had a very high copy number in yeast and was available in the laboratory. The disadvantages of this vector will be discussed later.

Subcloning is summarised in figure 5.2. The first step in subcloning was to produce double-stranded phage DNA for each mutant clone. Cells infected with the phage were grown up and a small-scale plasmid preparation was carried out. The double-stranded DNA was cut with HindIII and SalI, liberating the mutant GPM gene on a 1.3kbp fragment. After running part of the digest in an agarose gel to check for complete restriction, the DNA was purified by

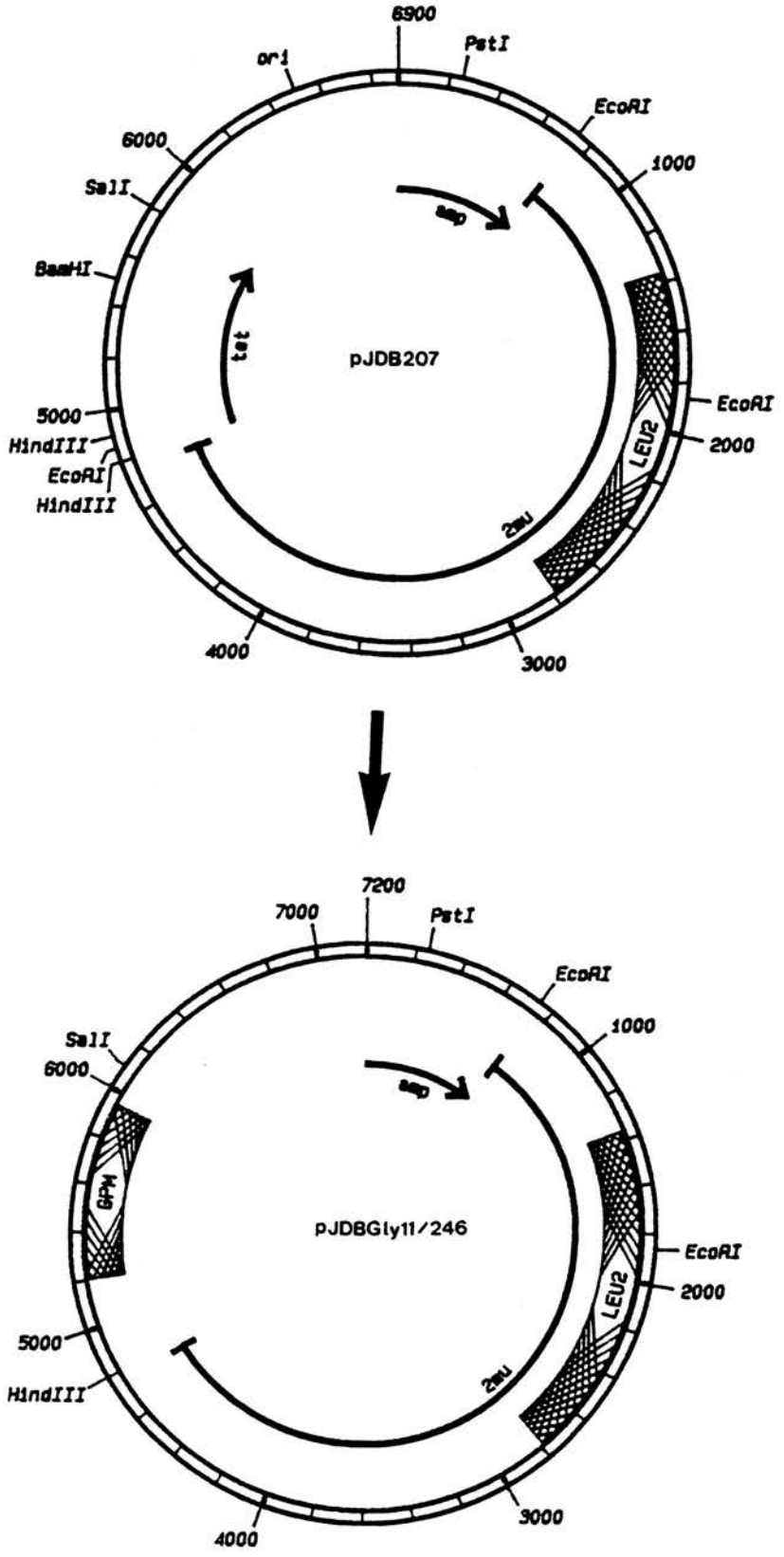

Figure 5.2. Subcloning strategy for the expression of the S11G and K246G mutants from the shuttle vector pJDB207.

phenol/chloroform extraction and ethanol precipitation. The shuttle vector pJDB207 was also cut with HindIII and SalI and the DNA purified. DNA from the two digests was mixed and ligated with T4 DNA ligase for 2 hours at RT, then transformed into competent TG1 cells and plated out on plates containing ampicillin. From the restriction maps of the vectors in figure 5.2, the transformants obtained would consist of the main fragment of pJDB207 with a HindIII-SalI insert from MW1 or from the plasmid itself. Transformants obtained from the ligations were grown up and plasmid DNA was isolated, then cut with the enzymes HindIII, SalI and BamHI and separated in an agarose gel. Clones which had received the small HindIII-SalI fragment of pJDB207 would contain one site for each enzyme, and thus give three bands on the gel. On the other hand, clones containing the GPM insert would have no BamHI site and would thus yield only two bands, one of 5.9 kbp corresponding to the vector and one to the 1.3kbp GPM insert. In this way plasmids consisting of the shuttle vector pJDB207 with inserts carrying each of the mutant GPM genes were obtained and identified. These plasmids were designated pJDBGly11 and pJDBGly246.

5.4 Transformation of DBYgpm- with pJDBGly11 and pJDBGly246

The next step towards the expression of the two mutant GPM enzymes in yeast was the transformation of DBYgpm- with pJDBGly11 and pJDBGly246. Maxi-preparations of the two plasmids were prepared on caesium chloride gradients, yielding about 500µg of each - more than sufficient for yeast transformation. Initially a simple LiAc

transformation of DBYgpm- was attempted, using 10μg of plasmid, and transformed cells were plated out on YOGE leu- plates to select for the ability to synthesise L-leucine, as the plasmids contained the LEU2 marker gene. Although in theory this protocol should yield up to 10^3 tranformants/ug DNA, no transformants were obtained from several repeated experiments. Clearly, one or more factors were adversely affecting the transformation efficiency. Several possibilities existed:

Firstly, it was possible that the strain DBYgpm- transformed poorly, as transformation efficiencies vary to a large extent from strain to strain. Although the parental strain DBY747 could be transformed without difficulty the deletion of the GPM gene, and the subsequent necessity to grow the cells using a glycerol/ethanol carbon source, might have altered the cell physiology, making transformation more difficult to acheive.

Secondly, the vector pJDB207 was known to possess a defective allele of the LEU2 gene, leu2d. This results in lower levels of expression of the gene product in cells containing this allele (Erhart and Hollenberg, 1983) and has two major consequences. On the plus side, the plasmid is forced to maintain a high copy number so that enough of the enzyme is produced; and this will result in high copy numbers for any extra genes inserted into the plasmid. On the minus side however, freshly transformed cells take much longer to attain a LEU+ phenotype and will not grow if challenged

with leu- conditions too soon after transformation. This drawback could be overcome by incubating transformants overnight in leu+ SOS media at 30°C before plating out.

Thirdly, the media used for the selection of transformants, YOGE leu- agar, constituted very poor growth conditions for yeast. This media was required in order to select for recipients of a LEU+ plasmid whilst not presupposing that any active phosphoglycerate mutase would be present. GPM+ LEU+ cells such as DBY747 transformed with YEP13.GPM grew very slowly on this media, so newly-transformed cells might have found it hard to establish growth at all, or might have grown very slowly indeed. If the mutant GPM genes carried by pJDBGly11 and pJDBGly246 coded for active enzyme, then transformants could be selected for the restored ability to utilise glucose by plating out on rich YD medium.

In order to address these three problems and to maximise transformation efficiency, the spheroplast transformation protocol of Burgers and Percival (1987, section 2.2.9.1) was used to transform DBYgpm- with 10μg of each plasmid. The transformants were kept at 30°C in SOS media supplemented with leucine overnight and then plated out, half in YD regeneration agar and half in YOGE leu- regeneration agar. Plates were incubated at 30°C until colonies were visible. The results are summarised in Table 5.1.

Table 5.1. Results of spheroplast transformation.

Plasmid	YD agar	YOGE leu- agar
pJDBGly246	7 colonies after 9 days.	0
pJDBGly11	0	2 colonies after 19 days.

5.5 Analysis of yeast transformants

The transformants obtained were first tested to ensure that they exhibited the correct phenotype. All grew on YD, YOGE leu- and YOD leu- plates and media, although the pJDBGly11 transformants took noticeably longer to grow on media containing glucose than cells containing either the wild-type or K246G phosphoglycerate mutase (see sections 5.6 and 5.7). In order to ensure that the transformants contained the expected plasmids, the plasmids were rescued from all the transformants and used to transform competent TG1 cells. In E.coli pJDB207 confers resistance to ampicillin. Resistant colonies were grown up and plasmid purifications were carried out. The plasmids obtained were then digested with HindIII and SalI and run in an agarose gel with size markers. In every case the restriction pattern observed matched that expected for pJDBGly11/246, i.e. two bands of 5.9 and 1.3 kbp (refer to figure 5.2). Finally, to ensure that the yeast transformants carried the

and one Gly246 transformant were denatured by heating and transferred onto duplicate Hybond-N filters using a slot-blot apparatus. As a negative control the plasmid YEP13.GPM, coding for wild-type GPM, was included; the plasmids pJDBGly11 and pJDBGly246 originally used to transform DBYgpm- were used as positive controls. The duplicate filters were then probed, one with labelled S11G oligonucleotide and the other with labelled K246G oligonucleotide. After hybridization, the filters were washed in 6xSSC at RT and autoradiographed for 4 hours at -70°C. The autoradiographs are shown in figure 5.3. Under these conditions hybridisation was quite nonspecific, and the oligonucleotides did not greatly differentiate between the various plasmids. The filters were then rewashed in 6xSSC at 47°C, which was 7 and 5°C below the Td's calculated for the oligonucleotides S11G and K246G respectively, and autoradiographed overnight at -70°C. At this stringency, the oligonucleotides would be expected to hybridise only to sequences which were a perfect match. As shown in figure 5.3, the oligonucleotide S11G hybridised only to the original and rescued pJDBGly11 plasmids whilst the K246G oligonucleotide hybridised specifically to the original and rescued pJDBGly246 plasmids.

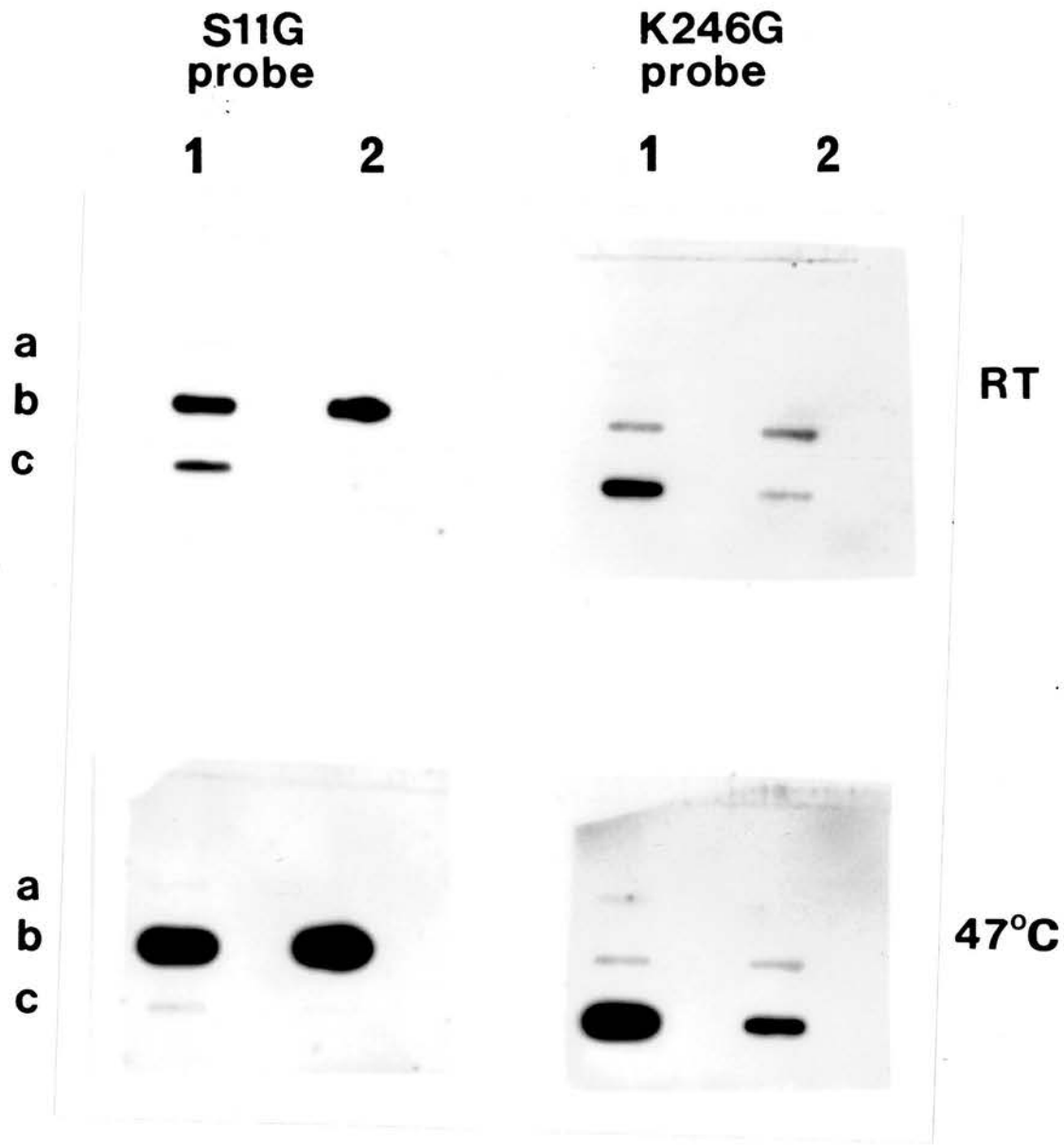

Figure 5.3. Slot-blot analysis of the S11G and K246G mutant GPM genes expressed from pJDB207 rescued from the yeast strain DBYgpm-.
a1 - YEP13.GPM; b1 - pJDBGly11; b2 - pJDBGly11 rescued;
c1 - pJDBGly246; c2 - pJDBGly246 rescued.
Experimental details are described in the text.

5.6 Expression levels of wild-type and mutant phosphoglycerate mutases

In order to assess the levels of expression of phosphoglycerate mutase in the yeast strain DBYgpm- transformed with YEP13.GPM, pJDBGly11 and pJDBGly246, crude cell extracts were analysed by SDS-PAGE. Figure 5.4 shows the levels of expression observed. Wild-type phosphoglycerate mutase is expressed from the vector YEP13 at fairly high levels, being the most highly expressed protein in the cell extract. This was also observed by Kawasaki and Fraenkel (1982) when YEP13.GPM was originally isolated by complementation, and is due to gene amplification caused by the multi-copy plasmid. The vector pJDB207 also has a high copy number in yeast, and the mutant S11G is overexpressed from the vector pJDB207 in DBYgpm-, although at around half the level observed for the wild-type enzyme. In contrast, the mutant enzyme K246G does not appear to have a high level of expression from pJDB207, and may be present at levels lower than those obtained from a single chromosomal copy of the wild-type gene (compare with figure 6.1)

Why are the two mutant enzymes, especially K246G, expressed at lower levels than the wild-type? The answer may lie in the length of yeast DNA present upstream of the GPM coding region. In YEP13.GPM several kilobases of upstream flanking sequence is present (figure 3.2), whereas for the constructs pJDBGly11 and pJDBGly246 only about 500 bases of upstream DNA are included (figure 5.2). It is possible that an upstream activating site (UAS) has been lost in these constructs, resulting in lowered

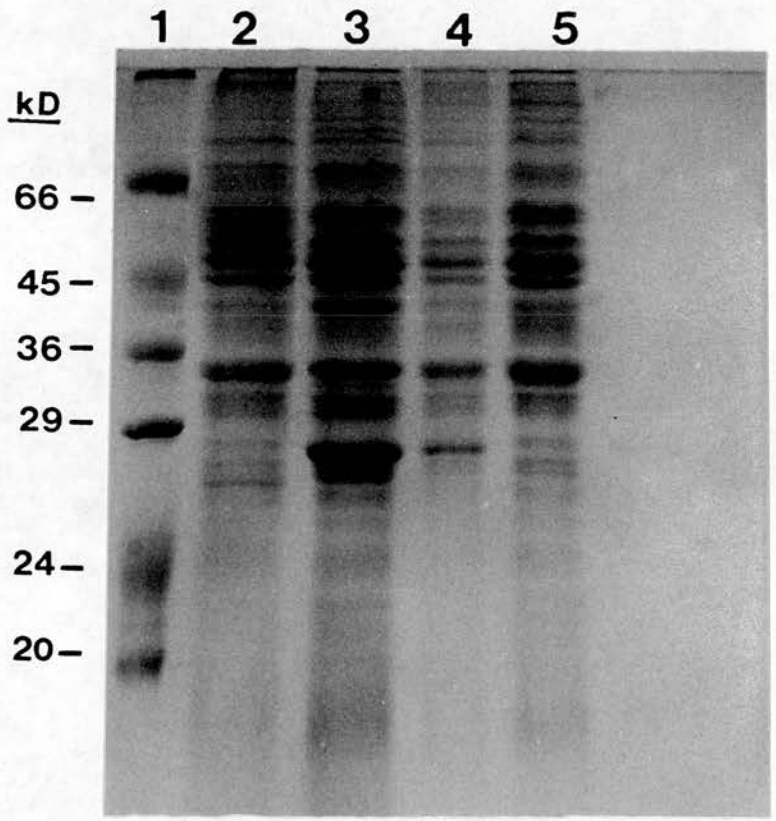

Figure 5.4. SDS-PAGE analysis of the expression of phosphoglycerate mutase.

1. markers
2. DBYgpm-
3. DBYgpm- + YEP13.GPM
4. DBYgpm- + pJDBGly11
5. DBYgpm- + pJDBGly246

expression levels. UAS's have been observed for several yeast genes, often considerably distant from the coding sequence (Guarente, 1984). This theory is supported by the work of Rodicio and Heinisch (1987), who found that expression of phosphoglycerate mutase from a multicopy plasmid decreased markedly when a region of DNA between the EcoRI and HindIII sites immediately upstream of the gene was removed (see figure 3.2 for restriction map). In the constructs pJDBGly11 and pJDBGly246, the upstream DNA terminates at the HindIII site, so it is possible that a UAS is present between the EcoRI and HindIII sites.

Why is the mutant K246G expressed at such low levels relative to the S11G enzyme, when both are expressed from the same plasmid, and have the same flanking sequences? It is possible that the particular clones chosen for the two mutants displayed differing expression levels due to variations in plasmid copy number, however the seven pJDBGly246 transformants screened all had very low levels of expression. One other possibility is that cells derive a selective advantage from the overexpression of the S11G mutant, for example if the enzyme had a very low activity. The inability to obtain pJDBGly11 transformants on media containing glucose could be due to the enzyme being barely catalytically competent. Yeast cells expressing the mutant enzyme S11G, and dependent on that enzyme for the utilization of glucose, were consistently observed to grow more slowly in media containing glucose than cells expressing the wild-type or K246G phosphoglycerate mutases. If this were the case then overexpression of the enzyme could confer a large selective advantage.

5.7 Growth characteristics

In order to further investigate the growth characteristics of the strain DBYgpm- expressing the wild-type and two mutant enzymes, growth curves were determined for the three strains in YD media (standard media containing glucose as carbon source) by measuring the increase in optical density at 600nm of triplicate 100ml cultures. Each culture was inoculated at time zero with an equal volume of a starter culture which had been grown to an OD_{600} of 1.0. The data are presented graphically in figure 5.5. Initially all cultures exhibited a lag phase; cells expressing the K246G enzyme were the first to enter exponential growth, followed closely by those expressing wild-type phosphoglycerate mutase. The slightly slower growth of cells with the wild-type phosphoglycerate mutase may be a consequence of the involvement of the synthetic machinery in maintaining the high level of expression of the enzyme observed in these cells. The strain expressing the S11G mutant displayed a considerably longer lag phase before entering log phase, but once in log phase appeared to be able to grow as quickly as the other strains. In a separate experiment, the level of expression of the S11G enzyme in DBYgpm- was analysed by SDS-PAGE during lag, exponential and stationary phases, and no significant changes were observed (data not shown). Further growth experiments together with measurements of the flux through glycolysis and of glycolytic intermediates are required to clarify these observations.

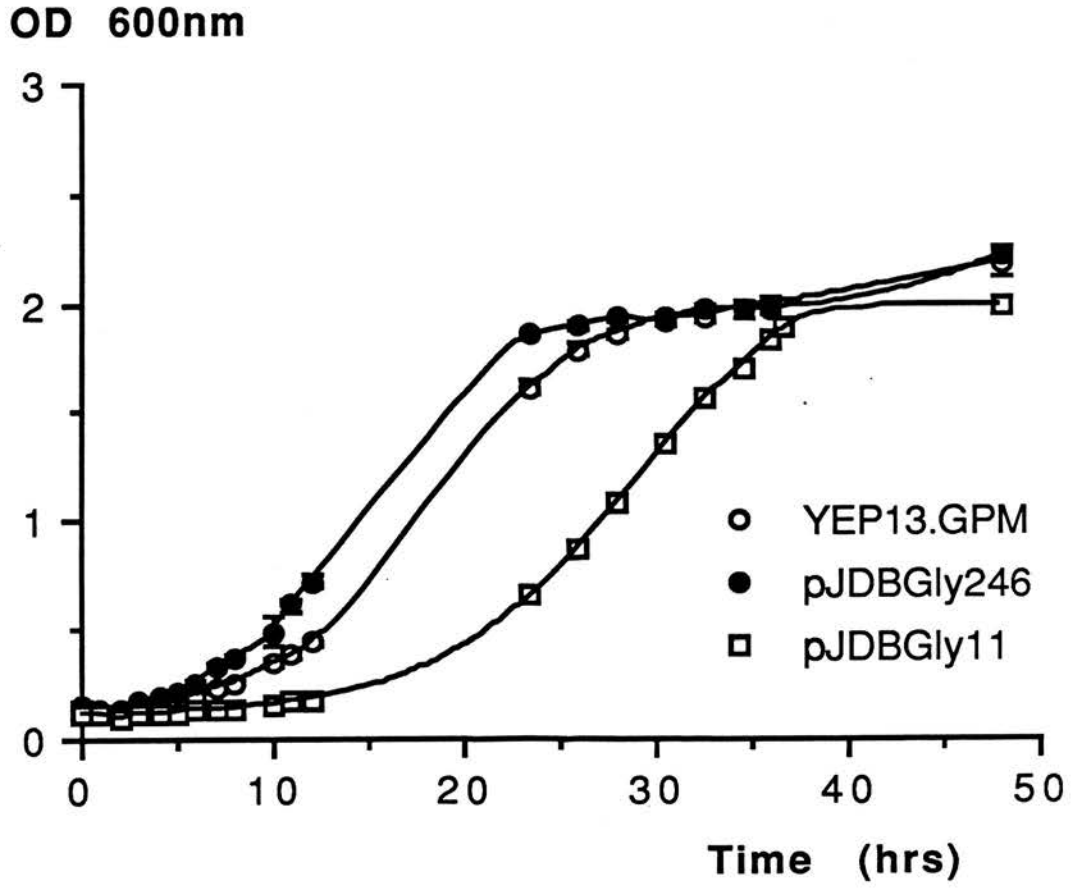

Figure 5.5. Growth curves for DBYgpm- transformed with YEP13.GPM, pJDBGly11 and pJDBGly246. Data points are the means of triplicates, and standard deviations are indicated.

In summary, two mutant forms of the enzyme phosphoglycerate mutase were designed, constructed and expressed in the yeast strain DBYgpm-. The fidelity of each mutation was confirmed in the respective transformed yeast cells, and expression levels were analysed. A preliminary investigation of the growth characteristics conferred by these enzymes was carried out. The purification and characterisation of the two mutant enzymes were now possible.

6. Purification of wild-type and mutant phosphoglycerate mutases

6.1 Background

The purification of yeast phosphoglycerate mutase was first reported over thirty years ago (Rodwell et al., 1956). Methods have continually been updated in line with advances in protein purification technology. The most recent relevant advance has been the introduction of affinity chromatography. It was discovered that the cofactor-dependent phosphoglycerate mutases, in common with many other glycolytic enzymes, would bind to a column of cibacron-blue Sepharose and could be specifically eluted using the substrates or cofactor. A final affinity chromatography step involving binding to cibacron-blue or other dyes such as cibacron-red-3BA (Pawluk et al., 1986) has been adopted in most of the more recent purification protocols for phosphoglycerate mutase from various sources including Leuconostoc (Kawai et al., 1981), S.cerevisiae (Price and Stevens, 1983), S.pombe (Price et al., 1985), pig heart (Pons and Carreras, 1985) and Z.mobilis (Pawluk et al., 1986).

6.2 Specific considerations

Historically, there has been no shortage of source material for the purification of yeast proteins. An obliging brewery could be relied on to provide yeast (an unwanted by-product) by the kilogram. Protocols for the isolation of yeast phosphoglycerate mutase typically start with several hundred grams wet weight of yeast and end up with several hundred mg of pure enzyme. For the purposes of this project, a much smaller scale of purification was both necessary and desirable. The use of transformed strains limited the amount of yeast

available to that which could be grown up in the laboratory; typically from 2-3 litres of culture at any one time. Hopefully, the overexpression of the enzyme from a plasmid would allow a higher yield of phosphoglycerate mutase per gram wet weight of cells. In any case, for the purposes of kinetic analysis as little as 300μg of pure enzyme would be sufficient. A simple, scaled-down protocol for the purification of wild-type phosphoglycerate mutase was thus devised.

6.3 Purification of phosphoglycerate mutase

Initially, cells of the yeast strain DBYgpm- transformed with the vector YEP13.GPM, and thus overexpressing wild-type phosphoglycerate mutase (figure 5.4), were lysed overnight in 1M ammonia according to the method of de la Morena et al., (1968). The lysate was then subjected to a 55-70% saturated ammonium sulphate fractionation, dialysed overnight, and applied to a cibacron blue-Sepharose column. After a NADH/AMP wash, phosphoglycerate mutase was eluted from the column by washing with a step of 10mM 3-PGA. When the purified protein was analysed by SDS-PAGE two bands of roughly equal intensity were apparent. The specific activity of the sample was about half that expected for pure phosphoglycerate mutase in the enolase-coupled assay (Price and Jaenicke, 1982). The two bands had apparent M_r's of 30,000 and 28,000. The more mobile protein exhibited the expected subunit size for yeast phosphoglycerate mutase and coincided with the protein overexpressed in crude extracts of yeast transformed with YEP13.GPM (figure 5.4). The contaminating protein thus appeared to be the larger of the two. The sample gave a single peak on HPLC (not shown) and yielded a single N-terminal protein sequence for the two

components corresponding to yeast phosphoglycerate mutase, indicating either an identical N-terminal sequence or a blocked N-terminus for the contaminating protein.

Proteolytic degradation during autolysis of yeast phosphoglycerate mutase has been well documented (Sasaki et al., 1976), and is thought to arise from the removal of successive amino acids from the C-terminus of the protein. The loss of about ten residues from the C-terminus of rabbit muscle phosphoglycerate mutase does not affect the binding or elution properties of the enzyme on a cibacron blue-Sepharose column (Price et al., 1983). However loss of all or part of the tail would be expected to yield smaller, more mobile bands on an SDS-PAGE gel whilst the contaminant in this case appeared to be larger.

Despite the anomalous observations from denaturing gels, the loss of the C-terminal tail during cell lysis remained the most likely explanation. Accordingly, a new method of cell lysis was introduced, as detailed in section 2.2.12.1 of the methods. The method was very fast, minimising the opportunities for proteolysis, and the protein was then purified as before. This procedure yielded a single band of M_r about 28,000 in SDS-PAGE (figure 6.1). The specific activity of the enzyme matched that reported by Price and Jaenicke (1982). The purification is summarised in table 6.1.

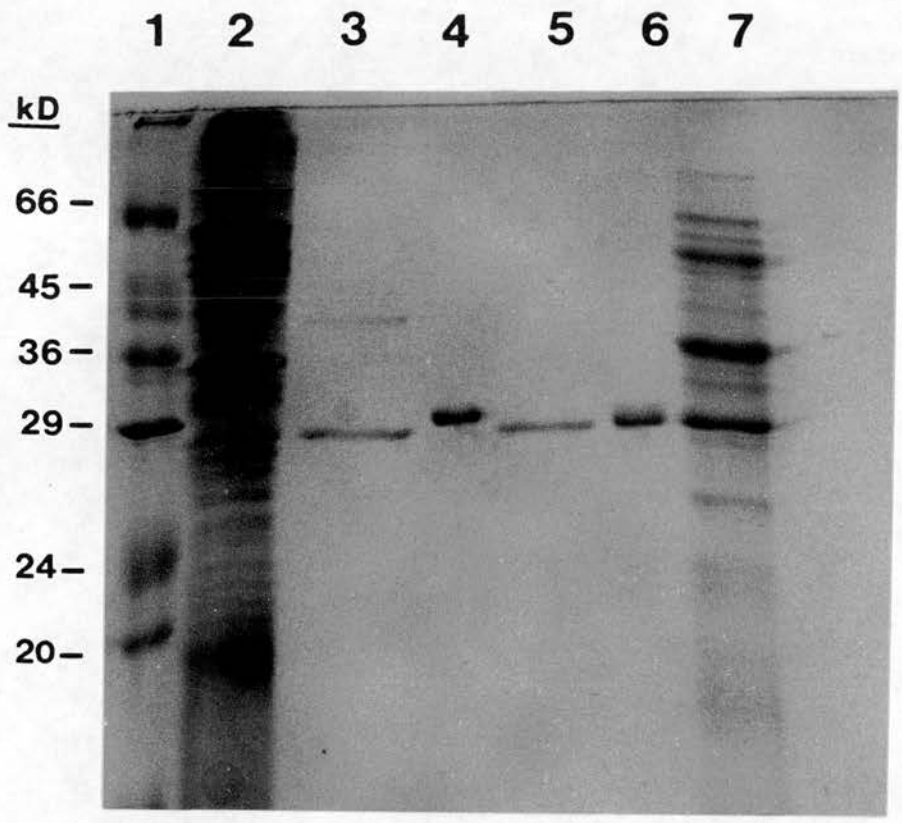

Figure 6.1. SDS-PAGE analysis of the purification of the wild-type and mutant forms of phosphoglycerate mutase from yeast.

1. markers
2. DBY747
3. purified K246G phosphoglycerate mutase
4. rabbit muscle phosphoglycerate mutase
5. pure wild-type phosphoglycerate mutase from modified procedure
6. autolysed wild-type phosphoglycerate mutase (two bands not clearly separated)
7. partly purified S11G phosphoglycerate mutase

Table 6.1. Purification of wild-type phosphoglycerate mutase.

The label 'crude' represents the initial cell extract; 'dial.' represents the sample after ammonium sulphate fractionation and overnight dialysis; 'column' represents the protein sample eluted from the cibacron blue-Sepharose column by 3-phosphoglycerate.

sample	volume (ml)	protein (mg)	EU	sp.act. (EU/mg)	yield (%)	purifn. (fold)
crude	82	3600	72200	20	100	1
dial.	2.0	77	59500	780	82	39
column	10.6	3.0	22500	7500	31	375

6.4 Purification of K246G

The mutant K246G was purified from the strain DBYgpm- transformed with pJDBGly246 using the same method as for the wild-type enzyme. As expected, the mutation introduced in the C-terminal tail did not alter the physical properties of the enzyme with respect to purification. Due to a much lower level of expression of this mutant (section 5.5 and figure 5.4), a lower yield of pure enzyme per g wet weight of cells was obtained. The purification of K246G is summarised in table 6.2. When analysed by gel densitometry, the enzyme appeared about 90% pure, with a minor amount of one contaminating protein (figure 6.1).

Table 6.2. Purification of K246G.

The samples are labelled as for table 6.1.

sample	volume (ml)	protein (mg)	EU	sp.act. (EU/mg)	yield (%)	purifn. (fold)
crude	86	1700	9240	5.4	100	1
dial.	3.3	85	2640	31	29	6
column	5.6	0.33	1960	6000	21	1100

6.5 Purification of S11G

The mutant enzyme S11G appeared to be significantly over-expressed in DBYgpm- transformed with pJDBGly11 (see figure 5.4). However, these cells grew significantly slower in YEPD medium than cells expressing either the wild-type or K246G enzymes. Purification of this mutant was at first attempted using the same method as for the other two enzymes, but problems of enzyme instability soon became apparent. Firstly, a drop in activity of about 20% per day was observed when the partly-purified enzyme was stored in 80% saturated ammonium sulphate at 4°C; conditions in which the wild type enzyme was stable indefinitely. The enzyme also lost a significant proportion of its activity when dialysed overnight against 10mM Tris-HCl pH7.5. The main problem however came at the affinity chromatography step. Despite repeated attempts, none of the enzyme activity loaded onto the cibacron blue-Sepharose column was ever recovered, neither in the wash-through nor in the NADH/AMP, 3-PGA or NaCl eluents. When samples of the eluents were run on SDS-PAGE, large amounts of protein

degradation products were observed. An attempt to further purify the protein by FPLC on a Superose-12 column also failed to yield any active enzyme. Because of the evident instability of the enzyme, and pressures of time, it was decided to use a partly-purified enzyme preparation for the kinetic analyses. In order to maximise the degree of purification whilst avoiding gel chromatography, a stepped ammonium sulphate fractionation was carried out. Cells were lysed, made 55% ammonium sulphate saturated, and spun as before, with the omission of the heating step. The protein was fractionated by successively increasing the degree of ammonium sulphate saturation by 5%, centrifuging, and assaying the specific activity of the enzyme in the pellets obtained. The purification table is shown below.

Table 6.3. Purification of S11G.

The sample labelled 'crude' represents the total cell extract whilst the other samples are denoted by the fraction of ammonium sulphate saturation in which they precipitated.

sample	volume (ml)	protein (mg)	EU	sp.act. (EU/mg)	yield (%)	purifn. (fold)
crude	53	345	200	0.58	100	1.0
55-60AS	0.5	14.3	8.5	0.59	4	1.0
60-65	0.7	13.6	34.	2.5	17	4.3
65-70	0.8	15.0	91.	6.1	46	11
70-75	0.8	15.2	102	6.7	51	12

The fraction with the highest specific activity was found to be that which precipitated between 70 and 75% saturated ammonium sulphate. It was resuspended in 80% saturated ammonium sulphate and 2,3-BPGA, which is known to stabilise the wild-type enzyme, was added to a final concentration of 1mM. When stored under these conditions at 4°C, the enzyme activity did not decrease significantly over several weeks. The enzyme was also stable under the conditions of the standard enolase-coupled assay for at least 30 min. Analysis by SDS-PAGE and densitometry showed that the S11G enzyme was about 25% pure in this fraction (see figure 6.1). This factor was taken into account in all subsequent kinetic experiments.

In summary, a procedure which allowed the rapid, small-scale purification of yeast phosphoglycerate mutase to homogeneity was developed and utilised to purify both the wild-type and K246G enzymes to allow kinetic analyses. The S11G mutant appeared to be inherently unstable, especially in dilute solutions, and only partial purification was achieved. The reason for this instability is unclear, but may be due to an increase in the flexibility of the polypeptide backbone caused by the introduction of a glycine residue.

7. Kinetic characterisation

This chapter describes the kinetic analysis of the purified wild-type and K246G enzymes and the partly-purified S11G enzyme. The kinetic parameters which were measured were:

a) The Km's for 3-PGA, 2-PGA and 2,3-BPGA.

b) The catalytic constants for the mutase, synthase and phosphatase activities.

All measurements for Michaelis constants were carried out in triplicate and standard deviations were calculated. Estimates of the Michaelis constants were made using non-linear regression analysis with weighting based on the standard deviations of each data point, using a program developed by Duggleby (1984). The data for the Michaelis constants are presented graphically as Hanes plots using the program CRICKETGRAPH on an Apple Macintosh microcomputer. The catalytic constants were calculated from three separate experiments assuming a subunit molecular mass of 27.5 kDa, and for the mutant S11G have been corrected based on a calculated purity of 25% obtained from gel densitometry.

7.1 Km for 3-phosphoglycerate

The Km for the glycolytic substrate 3-phosphoglycerate was measured using an assay coupled through enolase, PK and LDH to the oxidation of NADH as described in section 2.2.13.1. The concentration of the cofactor 2,3-bisphosphoglycerate was constant at 0.1mM for the wild-type and K246G, and at 0.2mM for the S11G enzymes. The enzyme activity was measured at five or six concentrations of

3-phosphoglycerate. The data are plotted in a Hanes plot (figure 7.1), and the estimates obtained for the Michaelis constants are summarised in table 7.1.

Table 7.1. Estimation of the Km's for 3-phosphoglycerate.

enzyme	Km 3-PGA (μM)	standard error	no. of data points
wild-type	510	± 60	6
K246G	590	± 100	6
S11G	720	± 40	5

The values obtained for the Km for 3-PGA of the wild-type and mutant enzymes agree well with the value of 710μM previously determined for the wild-type enzyme from yeast (McAleese et al., 1985).

7.2 Km for 2-phosphoglycerate

The Km for the gluconeogenic substrate 2-phosphoglycerate was measured using an assay coupled through PGK and GAPDH to the oxidation of NADH, as described in section 2.2.13.2. The concentration of the cofactor was as described in section 7.1 above. The enzyme activity was measured at five or six concentrations of 2-phosphoglycerate, and the data are plotted in a Hanes plot (figure 7.2). The estimates obtained for the Michaelis constants are summarised in table 7.2.

Table 7.2. Estimation of the Km's for 2-phosphoglycerate.

enzyme	Km 2-PGA (μM)	standard error	no. of data points
wild-type	52	± 7	5
K246G	72	± 5	6
S11G	59	± 16	5

These values agree with the estimate for the Km for 2-PGA of <100μM previously determined for the wild-type yeast enzyme (Rodwell et al., 1957).

7.3 Km for 2,3-bisphosphoglycerate

The Km for the cofactor 2,3-bisphosphoglycerate was determined using an assay coupled through the enzymes enolase, PK and GAPDH to the oxidation of NADH, as described in section 2.2.13.1. The concentration of the substrate 3-PGA was constant at 10mM. The enzyme activity was measured at five concentrations of 2,3-BPGA, correcting for the presence of low amounts of 2,3-BPGA in the enzyme samples and in the 3-PGA, and the data were plotted in a Hanes plot (figure 7.3). The apparent Km values obtained for the wild-type, K246G and S11G enzymes are summarised in table 7.3.

Table 7.3. Estimation of the Km's for 2,3-bisphosphoglycerate.

enzyme	Km 2,3-BPGA (μM)	standard error	no. of data points
wild-type	5.5	± 0.9	5
K246G	3.3	± 0.6	5
S11G	49	± 16	5

These compare with a value of 14μM previously determined for the wild-type yeast enzyme under similar conditions and with the same concentration of 3-PGA (Johnson and Price, 1987). At this concentration, 3-PGA will compete with 2,3-BPGA for binding sites on the enzyme, raising the apparent Km value for the cofactor. Using low, non-competitive concentrations of 3-PGA, the Km for 2,3-BPGA of the wild-type enzyme has been calculated as 0.8μM (Chiba et al., 1970).

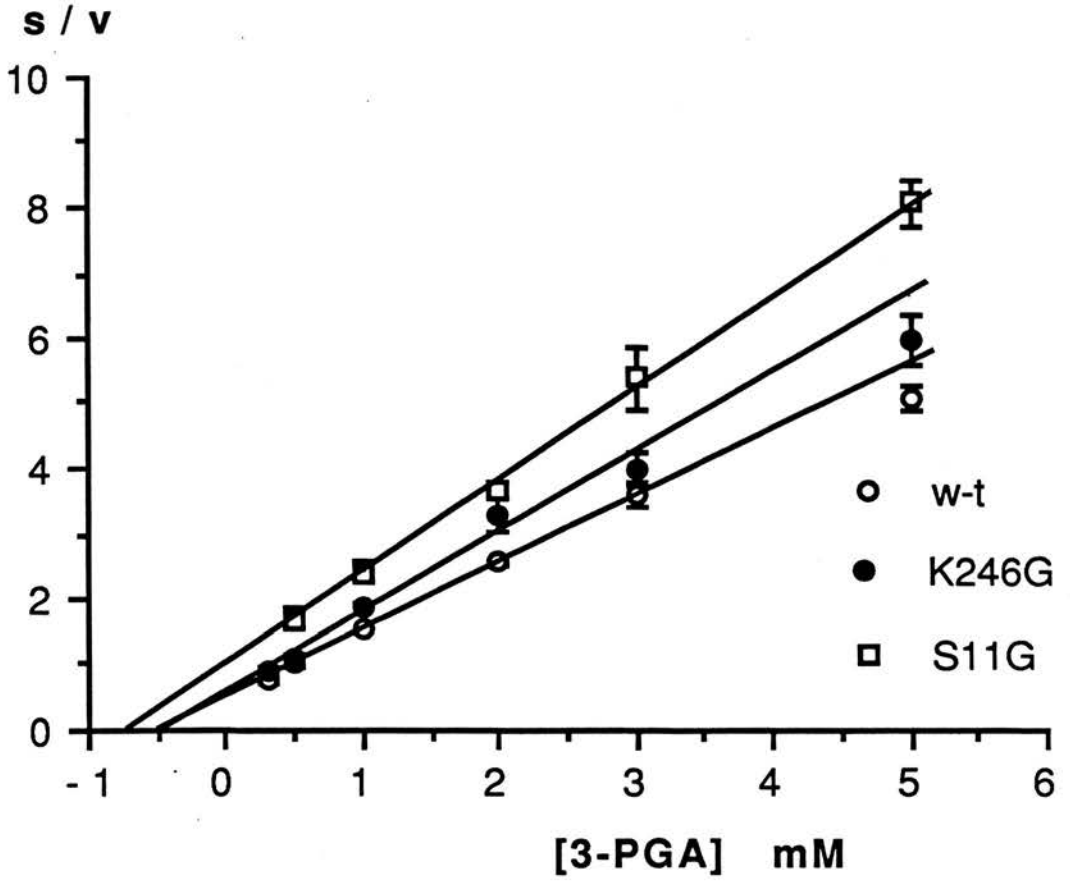

Figure 7.1. Hanes plot showing the Km for 3-phosphoglycerate of the wild-type, S11G and K246G forms of phosphoglycerate mutase. The points represent the means of triplicate measurements, and standard deviations are indicated.

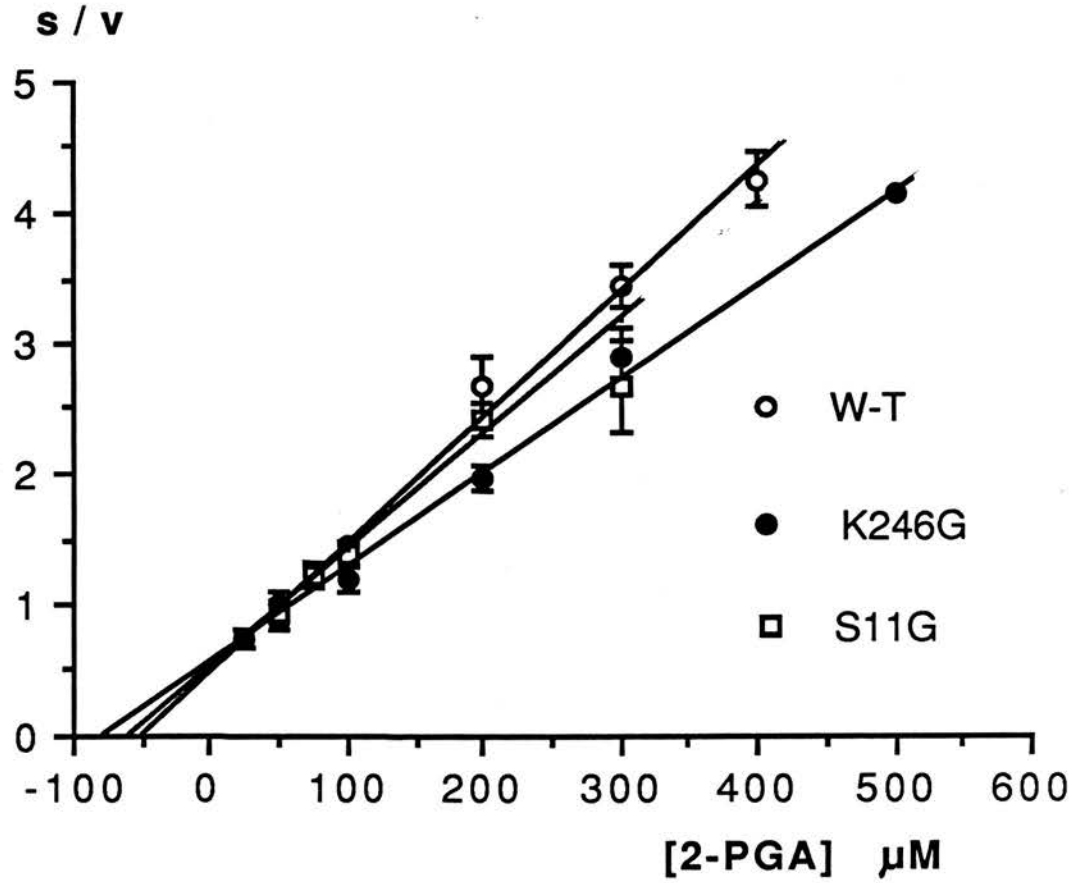

Figure 7.2. Hanes plot showing the Km for 2-phosphoglycerate of the wild-type, S11G and K246G forms of phosphoglycerate mutase. The points represent the means of triplicate measurements, and standard deviations are indicated.

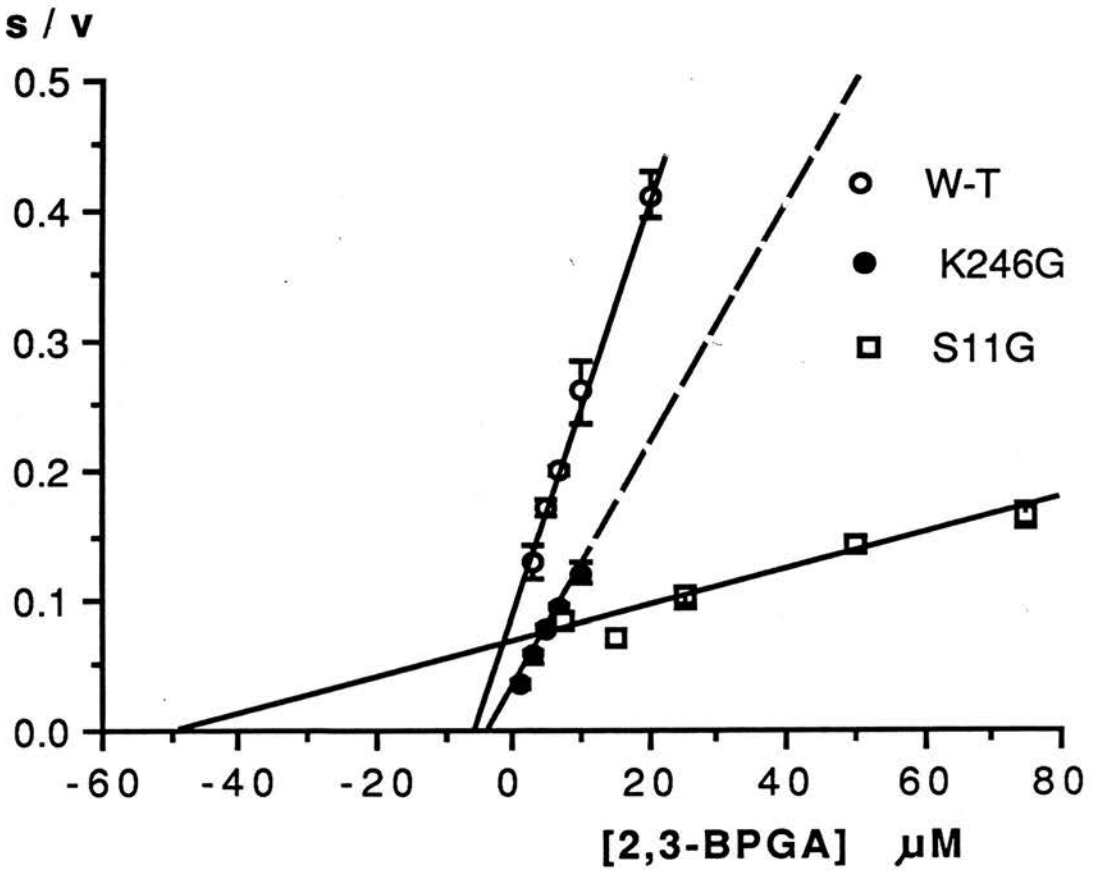

Figure 7.3. Hanes plot showing the Km for 2,3-bisphosphoglycerate of the wild-type, S11G and K246G forms of phosphoglycerate mutase. The points represent the means of triplicate measurements, and standard deviations are indicated.

7.4 Mutase activity

The catalytic constants of the three enzymes for the phosphoglycerate mutase activity in both the glycolytic ('forward') and gluconeogenic ('reverse') directions were determined using the assays described in sections 2.2.13.1 and 2.2.13.2, respectively. Maximal activity in the forward direction was observed with a concentration of 10mM 3-PGA, and in the reverse direction with a concentration of 400μM 2-PGA. At higher concentrations, substrate inhibition was observed, probably due to the competition of substrate with cofactor at the active site. The values obtained are summarised in table 7.4.

Table 7.4. Catalytic constants for the phosphoglycerate mutase reactions in the forward and reverse directions.

All values are the means obtained from three separate experiments, and standard deviations are indicated.

enzyme	catalytic constant (s^{-1})		ratio f/r
	forward	reverse	
wild-type	380 ± 10	520 ± 25	0.74
K246G	320 ± 34	420 ± 15	0.78
S11G	2.1 ± 0.1	1.0 ± 0.08	2.1

7.5 Synthase activity

The 2,3-bisphosphoglycerate synthase activities of the three enzymes were measured using an assay which continually regenerated the substrate 1,3-bisphosphoglycerate, thus avoiding the problems of instability of this molecule. The amount of 2,3-BPGA formed was estimated by measuring its ability to stimulate the phosphoglycerate mutase reaction (section 2.2.13.3). The values obtained in this way were assumed to represent maximal velocities for the synthase activities of the three enzymes, and are presented in table 7.5.

Table 7.5. Catalytic constants for the 2,3-bisphosphoglycerate synthase activity.

All values are the means obtained from three separate experiments, and standard deviations are indicated.

enzyme	catalytic constant (s^{-1})
wild-type	0.022 ± 0.0006
K246G	0.032 ± 0.0004
S11G	0.020 ± 0.003

These values are in good accordance with the only other reported measurement of yeast phosphoglycerate mutase synthase activity, of 0.02 s^{-1} (Sasaki et al., 1976).

7.6 Phosphatase activity

The 2,3-bisphosphoglycerate phosphatase activity of the three enzymes was determined in the presence and absence of the activator 2-phosphoglycollate as described in section 2.2.13.4, and are summarised in table 7.6.

Table 7.6. Catalytic constants for the 2,3-bisphosphoglycerate phosphatase reaction.

All values are the means obtained from three separate experiments, and standard deviations are indicated.

enzyme	catalytic constant (s^{-1})		stimulation
	basal	stimulated	(fold)
wild-type	0.0065 ± 0	0.080 ± 0.003	12
K246G	0.0067 ± 0.0004	0.100 ± 0.002	16
S11G	0.00049 ± 0.00003	0.0042 ± 0.0001	9

7.7 Wild-type phosphoglycerate mutase

The purified wild-type enzyme had kinetic characteristics similar to those previously reported for both yeast and mammalian phosphoglycerate mutases. The ratio of the mutase (forward) : synthase : phosphatase (basal) activities was 1000:0.06:0.02, which is in good agreement with the ratios previously reported of 1000:0.03:0.02 for yeast (Sasaki et al., 1976); 1000:0.1:0.06 for pig

muscle and 1000:0.06:0.05 for cat muscle phosphoglycerate mutase (Pons et al., 1985). The ratio for the mutase : phosphatase activities of the rabbit M-type enzyme has also been measured by ^{31}P-NMR, which yielded a ratio of 1000:0.06, confirming the kinetic results (Robinson et al., 1984). Calculation of the substrate specificity using the value k_{cat}/Km yields values of $7.5 \times 10^5 s^{-1} mol^{-1}$ for 3-phosphoglycerate and $1 \times 10^7 s^{-1} mol^{-1}$ for 2-phosphoglycerate. This indicates that as a substrate, 2-phosphoglycerate is preferred over 3-phosphoglycerate by a factor of 13.

7.8 K246G phosphoglycerate mutase

The mutant enzyme K246G displayed affinities for 3-PGA, 2-PGA and 2,3-BPGA which differed only slightly from those observed for the wild-type enzyme. The catalytic constants for the forward and reverse mutase reactions were about 15% lower than for the wild-type enzyme, whilst the synthase activity was slightly higher and the basal phosphatase activity about the same. The increased stimulation of the phosphatase activity observed for K246G in the presence of 2-phosphoglycollate may be significant, or may be a reflection of differing proportions of 'tailed' and 'tail-less' enzyme in the two preparations (see section 1.11). The ratio of mutase : synthase : phosphatase activities was 1000:0.1:0.02 - similar to that reported for other phosphoglycerate mutases. Overall, the loss of the C-terminal lysine appears to have had little effect on the kinetic parameters of phosphoglycerate mutase. The isolation and resequencing of the mutase gene from the yeast which have been used as a source of the mutant enzyme will be essential, in order to ensure that no

spurious mutations have occured. The possibility of contamination by another strain of yeast, which would result in a similar kinetic profile to that ascribed to the K246G enzyme, must also be rigorously excluded. With these provisos, our ideas about the functional significance of the tail in general, and the two C-terminal lysines in particular, may have to be revised.

7.9 S11G phosphoglycerate mutase

The replacement of serine 11 with a glycine residue appears to have resulted in dramatic changes in the kinetic characteristics of the mutant phosphoglycerate mutase. These are discussed individually below. It should be emphasised that the kinetic results are based on an enzyme which is only about 25% pure; obviously further purification will be necessary. Again, until the mutase gene is resequenced from the yeast which have been used as a source of the mutant enzyme, the possibility of spurious mutations cannot be ruled out, and the following discussion must be read with this reservation in mind.

a) Affinity constants

The affinity for the cofactor 2,3-bisphosphoglycerate appears to have been decreased by a factor of about ten with respect to the wild-type enzyme. This may confirm the suspected role of serine 11 in the binding of the cofactor, probably as a phospho-ligand for the transferrable phospho-group. As discussed in section 1.10.1, the affinities of E isoenzymes for 2,3-bisphosphoglycerate are much lower than those of their corresponding M and B isoenzymes. This could be mainly due to the lack of the serine 11 phospho ligand in the E

isoenzymes. The affinities for the substrates 2- and 3-phosphoglycerate appear little changed by the S11G substitution, suggesting that serine 11 plays no part in their binding at the active site of the enzyme. During catalysis, the mono-phosphorylated substrates are thought to bind to the catalytically active phosphorylated form of the enzyme (section 1.8.3). If this were the case, then serine 11 would not be expected to be involved in their binding.

b) Mutase and Phosphatase activities

The phosphoglycerate mutase activity of the S11G enzyme appears to have decreased by 99.5% for the forward direction and by 99.8% for the reverse direction with respect to the wild-type enzyme. This dramatic loss of activity does not appear to have been disastrous to the yeast strain expressing this enzyme, which can still utilise glucose as sole carbon source - although it does have a noticeably longer lag phase (section 5.7). Estimating that the enzyme is over-expressed by a factor of ten in this strain (fig. 5.4), then only 2-5% of the normal mutase activity is present in these cells. This observation points to a large excess of catalytic capacity for phosphoglycerate mutase in normal yeast cells.

The large drop in the mutase activity is probably due to a large decrease in the rate of phosphorylation of the enzyme by 2,3-bisphosphoglycerate. This rate has been shown to be very fast for chicken muscle phosphoglycerate mutase ($>1000s^{-1}$), but much slower for the horse erythrocyte bisphosphoglycerate mutase ($2.3s^{-1}$) (Rose and Dube, 1976). The catalytic constant of the S11G mutant for the mutase reaction of 2.1 s^{-1} agrees well with the observed rate of

phosphorylation of the synthase enzyme of $2.3s^{-1}$, suggesting that phospho-transfer from 2,3-bisphosphoglycerate to the enzyme may be the rate-limiting step in the mutase reaction catalysed by the S11G mutant.

Another intriguing observation is that the ratio of the maximal activities of the mutase reaction in the glycolytic (forward) and gluconeogenic (reverse) directions has shifted from f/r=0.74 for the wild-type enzyme to f/r=2.1 for the S11G mutant. This change can not be accounted for by a change in the relative affinities for 2- and 3-phosphoglycerate, which appear more or less unaltered. In the mechanism postulated for the mutase reaction, as described in section 1.9.1 and figure 1.8, catalysis is initiated by phospho-transfer from the enzyme to bound 3-phosphoglycerate (for the forward direction) or 2-phosphoglycerate (for the reverse direction). It could be possible that the S11G replacement has affected these two phospho-transfer steps to differing extents, making the transfer to 3-phosphoglycerate more favourable at high concentrations of substrate. It is worth noting that, using the value k_{cat}/Km as a measure of substrate specificity, 2-phosphoglycerate is still the more favoured substrate by a factor of about 6 over 3-phosphoglycerate.

The basal 2,3-bisphosphoglycerate phosphatase activity of the S11G mutant is only 8% of that observed for the wild-type enzyme. The rate of this reaction is much lower than that of the mutase reaction, and is therefore probably not limited by the common step of phosphorylation of enzyme by cofactor. It may instead be limited by the rate of phospho-transfer from the enzyme to an acceptor molecule such as water. The phosphatase activities observed for the chicken and human E isoenzymes are about 10% of those found in their

respective B isoenzymes (Bosch et al., 1982; Ikura et al., 1978).

c) Synthase activity

The 2,3-bisphosphoglycerate synthase activity of the S11G mutant does not appear to differ significantly from that of the wild-type enzyme. In this reaction, 1,3-bisphosphoglycerate phosphorylates the enzyme in a step which is probably rate-limiting for both B and E isoenzymes (Rose and Dube, 1976). The loss of the serine 11 ligand has therefore probably neither increased nor decreased the rate of phospho-transfer from 1,3-bisphosphoglycerate. As already mentioned (section 1.10.1), E isoenzymes have a tenfold higher affinity for 1,3-bisphosphoglycerate than B or M isoenzymes, giving rise to a correspondingly higher rate of phospho-transfer, and therefore synthase activity. Although no measurements have been made here, it is unlikely that the S11G enzyme possesses a higher affinity for 1,3-bisphosphoglycerate than the wild-type.

In summary, a preliminary analysis of the kinetic parameters of the wild-type and two mutant forms of yeast phosphoglycerate mutase has been carried out. The wild-type enzyme has kinetic characteristics similar to those found previously for both the yeast enzyme and the mammalian M and B isoenzymes. The K256G mutant does not appear to differ!significantly from the wild-type enzyme, suggesting that the C-terminal lysine is not essential for activity. The S11G mutant appears to display altered characteristics which may reinforce our ideas about the molecular basis for the mutase and synthase activities catalysed by the phosphoglycerate mutase family. Resequencing of the mutated genes in DBYgpm- remains an essential task.

8. General Discussion and Future Work

In the last few years, site-directed mutagenesis has increasingly been employed to further the degree of our understanding of the molecular basis for enzyme catalysis. The yeast enzyme phosphoglycerate mutase, for which the amino acid sequence and crystal structure are known; and for which a detailed reaction mechanism has been postulated; presents an ideal target for study by this method. In the work presented in this thesis, two aspects of the molecular structure/function relationship for yeast phosphoglycerate mutase have been studied. These conclusions drawn here must be qualified by the lack of incontrovertible evidence that the 'mutant' enzymes which have been studied are in fact identical to those designed. Possiblities remain that contamination or spurious mutations could account for the enzyme characteristics observed. Sequencing of the entire mutant GPM genes as expressed in the yeast from which the mutant enzymes have been isolated will be required.

Firstly, the importance of the C-terminal lysine residue in the flexible 'tail' of the enzyme has been probed by replacing it with a glycine residue to form the mutant enzyme K246G. On the basis largely of structural evidence, an important role has been ascribed to this lysine together with its sister lysine in the penultimate position at the C-terminus. The positive charges associated with these residues are thought a) to provide a phospho-ligand at the active site in order to stabilise the enzyme/transition state complex and b) to neutralise the charge associated with the carboxyl terminus of the enzyme. However, the mutant K246G appears to have kinetic properties very similar to those of the wild-type enzyme, indicating that lysine 246 may not be involved in the catalytic mechanism of the enzyme. The

possibility remains that lysine 245 is present at the active site and that the carboxy terminus forms a salt link with a residue near the active site, helping to anchor the tail during catalysis. Obviously this interpretation is open to examination by further site-directed mutagenesis.

Secondly, the close relationship of phosphoglycerate mutase with the enzyme bisphosphoglycerate mutase (its probable offspring) has been studied by the creation of a mutant form of the enzyme in which serine 11 has been replaced by a glycine (S11G). Bisphosphoglycerate mutase has evolved a tendency to release (and therefore synthesize) 2,3-bisphosphoglycerate in preference to the continual recycling of the compound which is favoured by its progenitor phosphoglycerate mutase. One of the differences observed at the active sites of the two enzymes is at residue 11 - a serine in phosphoglycerate mutase, where it is thought to help bind 2,3-bisphosphoglycerate but a glycine in bisphosphoglycerate mutase, where it can play no binding role. The mutant enzyme S11G appears to have a markedly reduced affinity for 2,3-bisphosphoglycerate which results in a low mutase activity, similar to bisphosphoglycerate synthase and a low synthase activity, similar to phosphoglycerate mutase. In other words, it may represent a 'halfway house' or 'hybrid' form of the two enzymes, and be catalytically incompetent to substitute for either. How could yeast phosphoglycerate mutase be made a better synthase? The crucial increase in the synthase activity observed for bisphosphoglycerate mutase must arise from its higher affinity for the substrate

1,3-bisphosphoglycerate, and this must be due to other residues near the active site. If further mutagenesis is to be contemplated with this aim in mind, then perhaps the best candidate is alanine 60, which is a serine in bisphosphoglycerate mutase. Would either an A60S mutant or a S11G/A60S double mutant possess an enhanced synthase activity? Again, site-directed mutagenesis can provide the answers.

In the near future, other aspects of the structure and activities of yeast phosphoglycerate mutase will be investigated by site-directed mutagenesis. Examination of the structural elements important in determining quaternary structure, and of the role in catalysis of other residues at the active site of the enzyme, will involve studies by crystallography, circular dichroism and protein NMR. Preliminary NMR experiments have indicated that the investigation of the function of the C-terminal tail using this technique may prove rewarding.

9. Summary

i) The gene encoding phosphoglycerate mutase from S.cerevisiae was localised on the plasmid YEP13.GPM supplied by Dr Dan Fraenkel by the use of oligonucleotide probes. The gene was subcloned into phage M13 and sequenced by dideoxy sequencing.

ii) The chromosomal copy of the GPM gene was deleted from the yeast strain DBY747 and replaced with the marker gene HIS3, creating the strain DBYgpm$^-$.

iii) Two mutant forms of the enzyme phosphoglycerate mutase, incorporating the replacements serine 11 to glycine (S11G) and lysine 246 to glycine (K246G) were designed and constructed using site-directed mutagenesis.

iv) The wild-type and two mutant forms of phosphoglycerate mutase were expressed in the GPM-deleted yeast strain DBYgpm$^-$ from shuttle vectors.

v) The wild-type and K246G enzymes were purified to homogeneity. Due to problems of instability, the S11G enzyme was only partly purified.

vi) Kinetic analyses of the three enzymes was carried out. The effects of the mutations introduced, and their significance towards an understanding of the structure/function relationship of phosphoglycerate mutase, have been discussed.

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Appendix A: Published paper

1. White, M.F. and Fothergill-Gilmore, L.A. (1988), 'Structure of the gene encoding phosphoglycerate mutase from *Saccharomyces cerevisiae.*', *FEBS Letts.* **229**, 383-387.

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Sequence of the gene encoding phosphoglycerate mutase from *Saccharomyces cerevisiae*

Malcolm F. White and Linda A. Fothergill-Gilmore

Department of Biochemistry, University of Edinburgh, George Square, Edinburgh EH8 9XD, Scotland

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The gene encoding yeast phosphoglycerate mutase was isolated, and its sequence was determined. The gene specifies a protein of 246 amino acids, and contains no introns. The sequence shows a strong codon bias. The upstream untranslated portion of the gene contains a CT-rich block such as is found in many highly expressed yeast genes, but does not have the associated CAAG sequence.

Phosphoglycerate mutase gene; Nucleotide sequence; *(Saccharomyces cerevisiae)*

1. INTRODUCTION

Phosphoglycerate mutases comprise a family of enzymes which catalyse reactions involving the transfer of phospho groups among the three carbon atoms of phosphoglycerates. There are at least five types of phosphoglycerate mutase that are kinetically and structurally distinct, but nevertheless have many features in common (e.g. see [1–3]). The enzyme from *Saccharomyces cerevisiae* has been extensively characterized, and the high-resolution crystallographic structure and amino acid sequence have been reported [4,5]. We present here the isolation and sequence determination of the gene encoding this enzyme, as a prerequisite for protein engineering studies to investigate its mode of action.

2. EXPERIMENTAL

The gene encoding *S. cerevisiae* phosphoglycerate mutase (GPM) has been obtained in the multicopy plasmid YEp13 by complementation [6], and was kindly given to us by D.G. Fraenkel. A restriction map of the plasmid was constructed, and the coding sequence was located with the use of oligonucleotide probes. A *Hin*dIII/*Sal*I restriction fragment containing the entire coding region together with several hundred base pairs of flanking sequences was subcloned into phage M13 for sequence determination by the dideoxy method. The use of the Sequenase DNA sequencing kit (US Biochemical Corp., Cleveland, OH) allowed sequences of about 500 bases to be determined from a single primer.

Correspondence address: L.A. Fothergill-Gilmore, Department of Biochemistry, University of Edinburgh, George Square, Edinburgh EH8 9XD, Scotland

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number X06408

3. RESULTS AND DISCUSSION

3.1. *Restriction mapping of YEp13-GPM*

Restriction mapping of the recombinant YEp13 plasmid revealed an insert of 6 kbp of *S. cerevisiae* chromosomal DNA (fig.1). The phosphoglycerate mutase coding sequence was located within this insert by Southern blotting with two end-labelled oligonucleotides corresponding to the N- and C-terminal regions of the protein sequence. Both oligonucleotides hybridized specifically to a 1.3 kbp *Hin*dIII/*Sal*I restriction fragment which was therefore subcloned into phage M13mp19 for sequencing.

3.2. *Nucleotide sequence of GPM gene*

The strategy used to sequence the phosphoglycerate mutase gene is summarised in

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fig.1. A contiguous stretch of 1154 bases was sequenced which includes an open reading frame of 741 bases encoding the enzyme phosphoglycerate mutase (fig.2). The DNA sequence largely agrees with the previously determined protein sequence, and the differences are discussed below.

Phosphoglycerate mutase is typical of glycolytic enzymes in yeast in being highly expressed. Like other highly expressed yeast genes [7], GPM exhibits an extreme codon bias. Only 31 codons are used, and of these 24 account for 93% of the amino acids (phosphoglycerate mutase has no cysteine residues). This compares with values of 94% for phosphoglycerate kinase, 95% for enolase I and 95% for alcohol dehydrogenase I. The upstream flanking sequence also has features similar to those of other highly expressed yeast genes. These include A residues at -1 and -3, and a 44 base A-rich region containing no G residues immediately adjacent to the initiating ATG codon [8]. Another feature found in many highly expressed yeast genes is a CT-rich block, here present at -83 to -109, but there is no associated CAAG sequence [9]. A TATA box at position -138 may be involved in the initiation of transcription, and the sequence CACACA at position -16 is found in several other yeast genes although its function is not known [9]. The downstream flanking region is AT-rich (73%), and contains two possible polyadenylation/transcription termination signals TAG...TAGT...TTT at positions 812 and 844 [10].

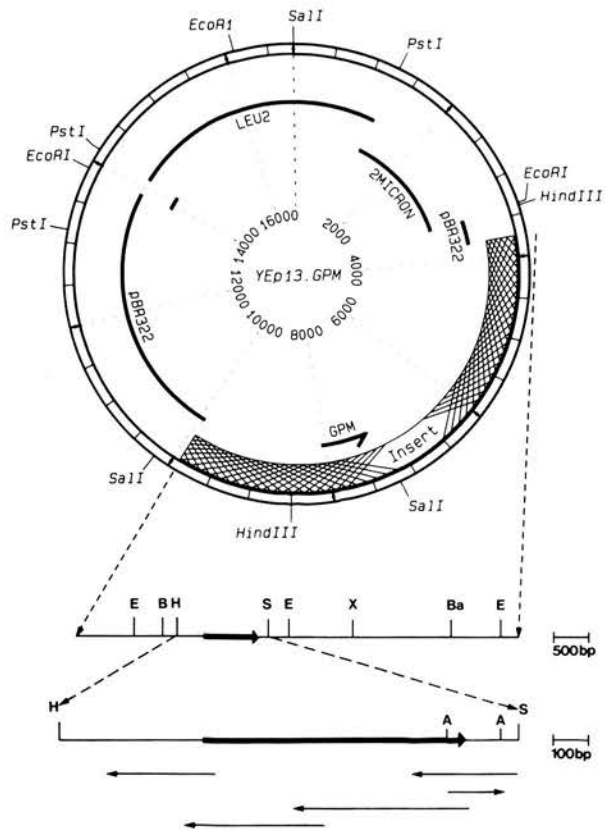

Fig.1. Restriction map of YEp13-GPM and sequencing strategy of GPM gene. The thick lines indicate the phosphoglycerate mutase coding sequence. Arrows show the direction and length of individual sequence determinations. Restriction enzymes: *Hin*dIII (H), *Sal*I (S), *Eco*RI (E), *Bgl*II (B), *Bam*HI (Ba), *Xho*I (X) and *Alu*I (A).

3.3. *Comparison of DNA and protein sequences*

A comparison of the amino acid sequence deduced from the DNA sequence with that determined by manual Edman degradation of proteolytic fragments of the protein [5] reveals several discrepancies (fig.2). Some of the differences probably relate to *S. cerevisiae* strain differences. The phosphoglycerate mutase used for the protein sequencing and X-ray crystallography was purified from dried baker's yeast, and was genetically heterogeneous as indicated by sequence microheterogeneities between residues 139 and 166 [5]. The sequence differences between residues 46 and 52 may indicate an additional heterogeneous region. The occurrence of sequence heterogeneities complicated the protein sequence determinations, and was the reason for wrongly assigning the position of the Asp-Leu sequence (residues 169–170). The other sequence discrepancies can be explained in terms of difficulties inherent in the manual Edman degradation procedure. For example, the amide assignments were largely based on the electrophoretic mobilities of peptides, and residue 99 was incorrectly assigned as glutamine because of the abnormally low pK of His-88. The interchange of the serine and valine residues at positions 172 and 176 resulted from having missed the labile serine residue after Edman degradation. Similarly, the sequence between residues 207 and 210 involves an incompletely cleaved proline and a labile threonine. The determination of the sequence of the C-terminal portion of the protein relied heavily

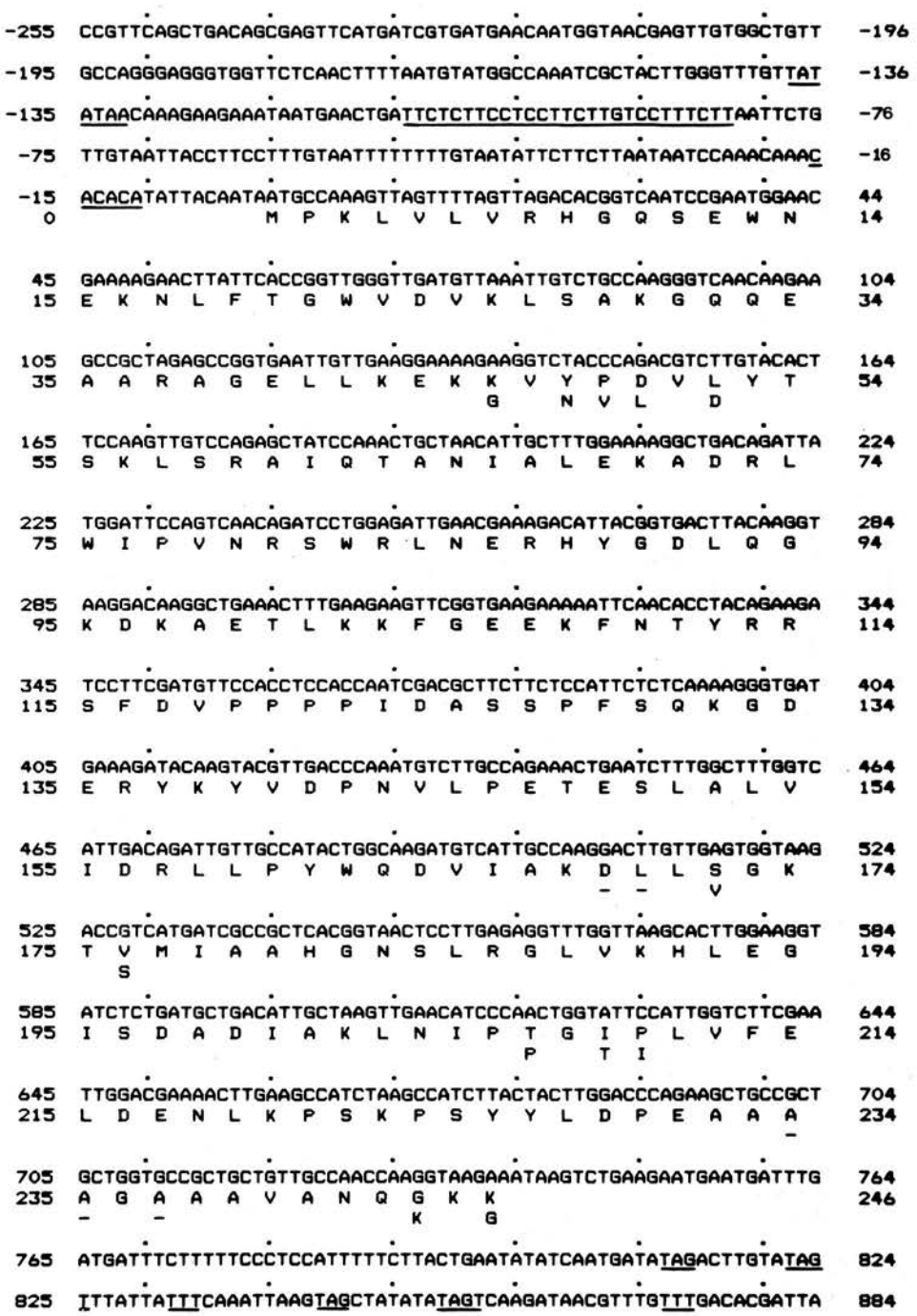

Fig.2. Nucleotide sequence and deduced amino acid sequence of GPM gene. The underlined sequences are discussed in the text. Residues different in the published protein sequence [5] are indicated below the deduced amino acid sequence, and are discussed in the text.

on overlapping peptic peptides, and the unusually large number of alanines between residues 232 and 239 (7 of 8 residues) was incorrectly identified. The presence of a glutamine at position 243 reduced the efficiency of Edman degradation, and the order of the C-terminal three residues was largely assigned on the basis of the complete resistance of this region to digestion by carboxypeptidases A, B and Y.

3.4. *Comparison with mammalian phosphoglycerate mutases*

Phosphoglycerate mutase exists as three different isoenzymes in mammalian tissues: type M

Fig.3. Comparison of amino acid sequences of yeast and mammalian phosphoglycerate mutases. The sequences are taken from the following references: human muscle phosphoglycerate mutase (HM) [14], human erythrocyte phosphoglycerate mutase (HE) [15], rabbit erythrocyte phosphoglycerate mutase (RE) [16] and fructose 2,6-bisphosphatase (FP) [13]. The numbering along the top of the sequences refers to the yeast sequence, and that below to the erythrocyte sequences. The boxed-in residues show the regions that are identical in three or more sequences. Gaps have been introduced in the Y and HM sequences to maximise homology.

predominates in muscle, type B in brain and type E in erythrocytes. The E type enzyme catalyses the synthesis and breakdown of 2,3-bisphosphoglycerate (2,3-BPG), in addition to catalysing the interconversion of 2- and 3-phosphoglycerates. This isoenzyme is frequently known as bisphosphoglycerate mutase, and plays a major role in regulating haemoglobin oxygen affinity as a consequence of controlling 2,3-BPG concentration. A comparison of the sequence of yeast phosphoglycerate mutase with the sequences of M and E type mammalian phosphoglycerate mutases is given in fig.3. These enzymes show the same slow rate of evolution as observed for other glycolytic enzymes [11], with about half of the residues identical between the yeast and mammalian enzymes. The two mammalian isoenzymes are as divergent from each other (49% different) as they are from the yeast enzyme, implying that the E isoenzyme experienced a period of rapid change after the gene duplication of an ancestral mutase gene before it settled down to the slow rate of change shown by a comparison of the rabbit and human E isoenzymes (3.5% different).

Most, but not all, of the active-site residues [4] are conserved. Thus, His-8 (yeast numbering) and His-181 which are involved in phospho transfer and Arg-59 which is required for binding the carboxyl group of the phosphoglycerates are identical in all sequences. However, Ser-11 and Thr-20 which in the yeast enzyme probably provide ligands for binding the transferred phospho group are not conserved. The replacement of Ser-11 by a glycine in the E isoenzyme is consistent with the greater instability of its phosphorylated form [12], and also with the relative ease with which the product 2,3-BPG can dissociate [1]. The replacement of Thr-20 by a cysteine explains why muscle but not yeast phosphoglycerate mutase is inhibited by thiol modifying reagents (review [11]).

The sequence of an active-site peptide from rat liver fructose 2,6-bisphosphatase has recently been reported, and its homology with the active site of phosphoglycerate mutases noted [13] (see fig.3). Like the mutases, fructose 2,6-bisphosphatase involves a phosphohistidine intermediate (His-8 of the yeast mutase), and the sequence similarities indicate that the enzymes have probably diverged from a common ancestor.

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